PHD

The effects of anti-inflammatory compounds on the oxidative metabolism of human phagocytic cells.

Maslen, Christina Louise

Award date:
1985

Awarding institution:
University of Bath

THE EFFECTS OF ANTI-INFLAMMATORY COMPOUNDS ON THE OXIDATIVE METABOLISM OF HUMAN PHAGOCYTIC CELLS.

Submitted by

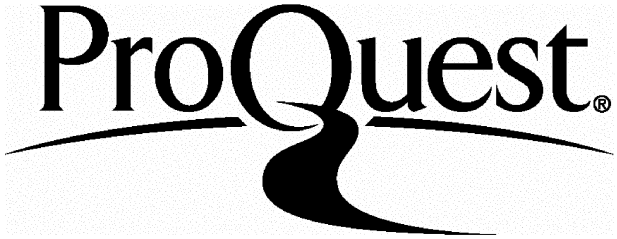
Christina Louise Maslen, B.Pharm.

for the degree of PhD

of the University of Bath

1 9 8 5

Christina Maslen



Rita: ..... He says there's a time for education. An' it's not when y' twenty-six an' married.

'Educating Rita' Willy Russell 1981

Act 1 Scene 8.

A C K N O W L E D G E M E N T S

I should like to thank my supervisor, Dr. Nick Hall, for his patience, guidance and many helpful suggestions when paralysis of the brain cell struck during the last three years.

I have calculated that, during the course of this work, I have used approximately 6.6 litres of blood. I am indebted to my friends and colleagues at BARC and RNHRD who so freely (well, fairly freely) gave their blood in the cause of nibbling away at the Frontiers of Science.

I should also like to acknowledge the help of Shirley Leader who trained me to take blood, only to find that my courage usually failed me and,uncomplaining,took over (much to the victim's relief). I must also include my grateful thanks to Cally Pettit who 'bled' all the NSAIDS in vivo patients.

I should like to thank Dr. Nick Goulding for guiding me through the intricacies of the Apple Computer (but I won't mention Voyage.....) and for his valuable help with statistics.

I gratefully acknowledge the financial support from the Science and Engineering Research Council over the last three years.

Last, but by no means least, I thank Annette Morris for her fast, accurate and conscientious typing of this thesis.

C O N T E N T S

A B B R E V I A T I O N S

cAMP	adenosine 3', 5' - monophosphate
ATP	adenosine 5' - triphosphate
pBPB	p-bromophenacyl bromide
C3, C3b, C3b', C4b, C5a, C5b	complement components
CGD	chronic granulomatous disease
CMFSS	calcium and magnesium free salt solution
con A	concanavalin A
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTNB	5,5' - dithiobis (2-nitrobenzoic acid)
EA	erythrocyte antibody
ETYA	eicosatetraynoic acid
FAD	flavin adenine dinucleotide
FMLP	n-formyl-l-methionyl-l-leucyl-l-phenylalanine
cGMP	guanosine 3', 5' - monophosphate
GSH	glutathione
HAGG	heat-aggregated immunoglobulin G
HETE	hydroxyeicosatetraenoic acid
HMP	hexose monophosphate
pHMPSA	p-hydroxymercuriphenylsulphonic acid
HPETE	hydroperoxyeicosatetraenoic acid
HRPO	horseradish peroxidase
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
LTB_4	leukotriene B_4
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate

NDGA	nordihydroguaiaretic acid
NSAIDS	non-steroidal anti-inflammatory drugs
$O_2^{-\bullet}$	superoxide anion radical
$OH^\bullet$	hydroxyl radical
OZ	opsonized zymosan
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
PRS	phenol red solution
PSS	progressive systemic sclerosis
RA	rheumatoid arthritis
SD	standard deviation
SH	sulphydryl group
SOD	superoxide dismutase
TMB-8	8 - (N, N-diethylamino) - octyl - 3,4,5 trimethoxybenzoate hydrochloride

SUMMARY

Phagocytic cells respond to activation by a variety of stimuli by generating oxygen-derived free radicals. The characteristics of the production of superoxide anion and hydrogen peroxide by human neutrophils has been compared with that produced by human monocytes.

The majority of work in this thesis is concerned with the employment of an assay which measures hydrogen peroxide produced by stimulated human neutrophils *in vitro*, but which also has been found to detect the generation of another peroxide, the identity of which is uncertain.

The use of cyclooxygenase, lipoxygenase and phospholipase A_2 pathway inhibitors has provided indirect evidence for the identification of the unknown peroxide as 5-hydroperoxyeicosatetraenoic acid (5-HPETE). These inhibitors have also provided the opportunity to investigate differences in the oxidative metabolism of human neutrophils induced by various stimuli. In addition, the effects of two cyclooxygenase inhibitors, diclofenac and piroxicam, on neutrophil activity *in vivo* has been investigated. Whilst neutrophil activity of some individuals was inhibited, this was not consistent and not significant. Incubation of a variety of analogues of the cyclooxygenase inhibitors diclofenac and fenclofenac with stimulated neutrophils *in vitro* has allowed an insight into the structure-activity relationships of these drugs' effects on neutrophil activity. It was found that the position of substitution of various groups in the ring structure remote from the acid group had the biggest single influence on activity.

Finally, the oxidative metabolism of neutrophils from patients with progressive systemic sclerosis, rheumatoid arthritis and peripheral vascular disease has been compared with that of neutrophils from healthy controls. The neutrophils from the progressive systemic sclerosis group were found to have increased activity both *ex vivo* and following

incubation with heat-aggregated IgG. This has been shown to be associated with enhanced expression of Fc receptors on these cells.

CHAPTER ONE

INTRODUCTION

INTRODUCTION

In 1882, the Russian scientist Elie Metchnikoff, using living, transparent invertebrates for his microscopic studies, discovered that there were cells capable of engulfing foreign matter, including bacteria. He called this process phagocytosis, literally, "cellular eating". It was he who realised that these phagocytic cells were in fact protecting the organism and that the acute inflammation which occurred at the same time that they appeared was essentially a curative response.

The aim of this Introduction is to give an outline of the phagocytic cells (chiefly the neutrophils as it is with these cells that the majority of the practical work of this thesis is concerned), the mechanisms by which they become activated and the consequence of that activation. This is followed by an appreciation of the role they play in the inflammatory response and in rheumatic diseases, and how non-steroidal anti-inflammatory drugs may modify their activity.

1. THE NEUTROPHIL

Neutrophils are short-lived end-cells with a high degree of functional specialization.

1.1 Structure

Neutrophils in the bloodstream measure approximately 12-15μm in diameter. The darkly-staining nucleus is divided into several sausage-shaped lobes which vary in number from 1 to 5. These lobes are connected to one another by very thin strands of nuclear material (Hirsch, 1974; Lisiewicz, 1980). This lobulated structure of the nucleus is thought to facilitate exit from the blood vessels as the cell can then move through the tight junctions between adjacent

endothelial cells (Segal, 1981). The cytoplasm contains large numbers of round or rod-shaped, membrane-bound granules of two types known as azurophil and specific granules. Most of these are usually gathered in the centre of the cell between the nuclear lobes. Neutrophils also have an abundant flowing plasma membrane with numerous 'ruffles' and pseudopods. They are lacking in endoplasmic reticulum and mitochondria, but have a microfilamentous network of actin and myosin and their related proteins (Segal, 1981).

1.2 Production and Fate

In the adult, neutrophils are produced mainly in the bone marrow and arise from a population of stem cells which are also the progenitor cells of monocytes, erythrocytes, eosinophils and basophils (Quesenberry and Lievitt, 1979). Maturation of the cell proceeds through the myeloblast, promyelocyte, myelocyte and metamyelocyte stages to juvenile forms and finally the mature marrow neutrophils. The proliferative stages take about 6 days to complete and maturation a further 6 days. Granulopoiesis is regulated by both stimulatory and inhibitory factors. The stimulatory factors (granulopoietins) include colony-stimulating factors from mononuclear phagocytes, activated lymphocytes and the vascular endothelium. Inhibition of the maturation of neutrophils has been noted following exposure to interferons, chemotactic peptides and lactoferrin (Palmblad, 1984). About 10^{11} neutrophils are produced in a normal adult every day (Dancey et al, 1976) and at any one time in the bloodstream there are about 60 x 10^{9} neutrophils. Half of these cells are circulating, the other half marginate to the lining of the vessels. The two populations appear to be in dynamic equilibrium. After about 6 to 7 hours in the circulation the cells move into the tissues. Neutrophils marginate and adhere to

the endothelium. This adherence may be augmented in inflammatory states by a plasma factor (Lentnek, Schreiber and MacGregor, 1976) and can be inhibited by anti-inflammatory agents (MacGregor, 1976). Following adherence the neutrophils crawl between adjacent endothelial cells (diapedesis) (Grant, 1974). In the tissues they survive for a few days at most. Approximately 10^{11} neutrophils (which represent 100g) must disappear from an adult's tissues each day (Palmblad, 1984). The methods by which they are destroyed and expelled are poorly understood. Suggested sites include lungs, liver, spleen, urine, saliva and digestive tract (Lisiewicz, 1980).

1.3 Locomotion and Chemotaxis

Locomotion is similar whether the cells are moving randomly or exhibiting chemotaxis. The cell has a pseudopod projected in front, a mid-region containing the nucleus and a knob-like tail. The pseudopod consists solely of cytoplasm and its protrusion is the first phase of locomotion. The second phase involves the translocation of the cell nucleus. The pseudopod pulls first the granules and then the nuclear segments from the remaining cytoplasm (Zigmond, 1978). Electron microscopy studies have revealed a complex arrangement of actin and myosin microfilaments just beneath the plasma membrane (Snyderman and Goetzl, 1981). These microfilaments are critical for directed locomotion and the attendant changes in cellular shape, whereas microtubule rearrangement is required to initiate and stabilize cellular orientation toward a chemotactic gradient. Cells treated with agents that disrupt microtubules, such as colchicine, exhibit a disorganised, non-directional type of movement (Palmblad, 1984).

Chemotaxis can be defined as the ability of motile cells to recognise and respond to a suitable concentration gradient with directional

migration. The classic assay system for chemotaxis was devised by Boyden in 1962. He used a two compartment chamber which demonstrated the movement of neutrophils through a filter into the compartment containing the chemotactic agent. This does not however differentiate between true chemotaxis and accelerated random locomotion stimulated by that chemotactic agent. Observing and measuring the movements of individual cells microscopically gives a more accurate picture. Chemotactic factors include products of complement activation (C3a, C5a), kallikrein, plasminogen activator, leukotriene B_4 and in vitro, the synthetic peptide n-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP). It has been suggested that there is a latent plasma factor which when activated by superoxide anion, becomes a powerful chemotactic agent (McCord, 1980). Certainly in models of inflammation induced in laboratory animals intravenous administration of superoxide dismutase, the enzyme which scavenges superoxide, prevents the accumulation of neutrophils at the inflammatory locus. This plasma factor does not stimulate degranulation or further superoxide production and hence does not perpetuate its own existence, but acts only as a chemotactic factor, recruiting more cells to the inflammatory lesion. However, other workers have not been able to demonstrate the existence of this factor. Another chemotactic factor for neutrophils is derived from these cells and is induced by crystals. Specific binding sites on the cell surface have been demonstrated for this glycoprotein (Spilberg and Mehta, 1979). Chemotaxis is initiated by the binding of chemoattractants to receptors on the cell surface. One means of modulating the sensitivity of cells to chemoattractants would be to alter the number, affinity or distribution of receptors along the surface of neutrophils during the chemotactic response and there is evidence that this does happen (Snyderman and Goetzl, 1981).

Inhibitors of chemotaxis, which serve to keep neutrophils at the inflammatory lesion once they have arrived include the peptide tuftsin; neutrophil immobilizing factor and α_2 macroglobulin (Goetzl, 1975). In fact, very high concentrations of chemoattractants themselves immobilize neutrophils (Zigmond, 1978). In vitro FMLP may trigger its own oxidative inactivation (Clark, 1982). Myeloperoxidase,hydrogen peroxide and a halide destroy the biological activity of the peptide by oxidation of a methionine residue in an example of a negative feedback control mechanism .

1.4 Phagocytosis

Attachment of a particle to receptors on the cell surface brings adjacent receptors into contact with that particle. This sequential attachment of adjacent receptors results in the plasma membrane being pulled up and round the particle to be ingested in what has been referred to as a "zipper-like mechanism". This is powered by the microfilamentous structures just below the surface of the membrane (Stossel, 1974). The phagocytic vacuole which contains the particle in a closed sac of inverted plasma membrane is formed when the pseudopodia fuse, with subsequent breakage of the neck of the membrane that links the phagocytic vacuole with the surrounding plasma membrane (Segal, 1981). The fate of the ingested particulate matter is closely linked to the process of degranulation and bactericidal killing, both of which are discussed later in this Introduction.

1.5 Neutrophil Surface Receptors

The receptors which neutrophils carry on their surface are probably glycoproteins, with their protein part embedded in the plasma membrane and the carbohydrate moieties sticking out into the extracellular fluid

acting as specific recognition probes (Roos, 1981).

Neutrophils bear receptors for the Fc fragment of immune complexes and aggregates formed by all IgG and IgA subclasses. They do not have receptors for IgM (Henson, 1977). Monomeric immunoglobulin binds only weakly to neutrophils although some workers have suggested specific receptors for aggregated and monomeric immunoglobulins (Henson and Oades, 1975; Treadway et al, 1979).

Neutrophils also bear receptors for complement components. The best studied of these are the receptors for the C3b and C3b' fragments, although there are also receptors for C4b; C5b and C5a; small C3 fragments and $\overline{C567}$ (Henson, 1977). Neutrophils carry more Fc receptors than C3b receptors (Hed, Stendahl and Sundqvist, 1982). Particles are phagocytosable if opsonized by IgG. IgG binds to Fc receptors and C3b acts as an amplification in the system. C3b fragments actually bind to the Fc portion of IgG and thus accelerate the ingestion of IgG-coated particles by binding to their own C3b receptors (Roos, 1981). Studies with coated particles show that coating with C3b only, although causing membrane perturbation, primarily promotes attachment of the particle, whereas coating with IgG primarily affects the ingestion phase. IgG-coated particles are 3-4 times more efficient at initiating activation of cells than C3b-coated particles (Hed and Stendahl, 1982). Wright and Silverstein, (1983), have also reported that while IgG-coated particles promote a vigorous release of hydrogen peroxide by neutrophils, C3b- and C3b'-coated particles do not.

These studies favour the concept that neutrophils possess different types of ingestion and activation mechanisms. One mechanism is represented by an IgG-mediated interaction where the antibody molecules, exposing their Fc-portions, become available to interact with abundant Fc-receptors on the neutrophil membrane. The initial Fc-

mediated interaction at the neutrophil surface is accompanied by a metabolic activation due to an interaction between Fc receptors and the enzyme responsible for oxidative metabolism. As there is no clear correlation between C3b-mediated attachment and metabolic activation, the receptor for C3b must show a lower association with the enzyme responsible for oxidative metabolism, (Hed and Stendahl, 1982; Hed, Stendahl and Sundqvist, 1982). The fact that opsonized zymosan does stimulate oxidative metabolism in vitro may be due to the fact that it is also coated with some IgG as well as with C3b (Hed and Stendahl, 1982). Binding of C3b-coated particles to C3b receptors can be completely inhibited by the addition of EDTA, or by the omission of calcium and magnesium salts from the incubation medium (Roos et al, 1981). However, the presence of divalent cations does not seem to be necessary for the binding of IgG-coated particles to Fc receptors (Roos et al, 1981). The function of complement receptors may be modified by the impermeant stilbene disulphonic acids (Tauber and Goetzl, 1981) as demonstrated by the suppression of superoxide production following stimulation of neutrophils pre-treated with this agent by opsonized zymosan, and by the inhibition of the expression of neutrophil C3b receptors as assessed by a rosetting assay. The fact that Tauber and Goetzl have found that opsonized zymosan can stimulate neutrophil oxidative metabolism, mediated via C3b receptors, supports the theory that the receptor for C3b must show a lower association with the enzyme responsible for oxidative metabolism (Hed and Stendahl, 1982) rather than an inability for C3b receptors to mediate oxidative metabolism at all (Wright and Silverstein, 1983). Many other receptors have also been demonstrated on neutrophils. Receptors are expressed for the chemotactic peptide FMLP (Williams et al, 1977). The number and affinity of these receptors is increased at

low sodium concentrations in the medium. This could be due to the collapse of the transmembrane potential that occurs when neutrophils are suspended in a sodium-free, high potassium medium. The transmembrane potential maintains the membrane proteins in a certain configuration so that any change could result in new configuration states of membrane proteins (De Togni et al, 1983). There are also receptors for the tumour promoter, phorbol myristate acetate (Lehrer and Cohen, 1981); specific mannoside-containing receptors for concanavalin A (Pick and Keisari, 1981); receptors for leukotriene B_4 (Kreisle and Parker, 1983); cytoplasmic receptors for steroids (Jones, Morris and Jayson, 1983); for prostaglandin (Weissmann, 1983) and for adenosine (Cronstein et al, 1983). Occupation of these receptors stimulates the cell independently of phagocytosis.

2. MONONUCLEAR PHAGOCYTES

In contrast with neutrophils, these phagocytic cells are long-lived and capable of differentiation and specialization.

2.1 Structure and Function

Monocytes originate from the same progenitor cells as do neutrophils. These stem cells in the bone marrow give rise to the promonocyte which in turn gives rise to the monocyte which, after a short maturation phase is released into the peripheral blood where it has a half-life of 22 hours before passing into the tissues (Quesenberry and Lievitt, 1979). The monocyte is about 14μm in diameter, has an indented nucleus and a moderate amount of cytoplasm which contains azurophilic granules. Monocytes have distinctive ruffled membranes

which are continually in motion and which are involved in the formation of pinocytic vacuoles (Steinman and Cohn, 1974).

The vast majority of macrophages which accumulate in inflammatory areas are of monocyte origin. They can remain in tissues for several months in a relatively quiescent state as resident cells. Once activated they can be categorized into a) Inflammatory macrophages, which are those stimulated by non-specific inflammatory factors, and are high in secretory activity but do not possess enhanced microbicidal or tumoricidal activity and b) Activated macrophages, which are those stimulated by immunologic factors via lymphocyte-derived products, and are low in general secretory activity but possess high activity in secreting reactive metabolites of oxygen and thus have microbicidal and tumoricidal activity (Takemura and Werb, 1984). Macrophages are larger than monocytes (20-100μm in diameter). Their nuclei vary from spherical to deeply indented structures. The cytoplasm contains granules and vacuoles. The plasma membrane shows increased activity, expressed in an increasing number of surface projections (Steinman and Coln, 1974).

As maturation of the mononuclear phagocyte proceeds, the number of enzyme-containing granules increases, and hence the macrophage contains more enzymes than the monocyte. The level of enzymes differs amongst macrophages with an increase indicative of activation. The enzymes include many acid hydrolases; neutral proteases such as elastase, collagenase and plasminogen activator, complement components and lysozyme. Macrophages also secrete enzyme inhibitors such as α_2-macroglobulin as well as a variety of factors that regulate functions of other cells such as interferon and interleukin 1 (Takemura and Werb, 1984).

Macrophages are able to phagocytose a much wider spectrum of material than neutrophils and can ingest particles smaller than 0.1μm whereas the neutrophil is ineffective at this size range.
The ultimate fate of macrophages is not known. It is likely that they are lost through the respiratory and digestive tracts, or that they are injured and phagocytosed by other macrophages (Steinman and Cohn, 1974).

2.2 Mononuclear Phagocyte Surface Receptors

Human monocytes carry receptors for the Fc portion of IgG. Studies have suggested that in fact the human monocyte possesses two types of Fc receptor, one of high affinity binding monomer and complexes, and one of low affinity which predominantly binds complexes (Carter, Leslie and Reeves, 1982; Rasmussen et al, 1983). Monocytes also carry receptors for C3b and C3b', which function independently of one another on a single cell. They do not have receptors for C3d. C3b and C3b' receptors do not normally mediate ingestion. However, if monocytes are first activated e.g. by phorbol myristate acetate, then ingestion does occur. The C3b' receptor is three times more efficient in promoting attachment and ten times more efficient in promoting ingestion than the C3b receptor (Wright and Silverstein, 1982). As reported for neutrophils, neither the C3b nor the C3b' receptor causes the release of oxygen metabolites from monocytes (Wright and Silverstein, 1983).

3. NEUTROPHIL ACTIVATION

3.1 Stimuli

Neutrophil activation *in vivo* is designed to ensure the ingestion and killing of micro-organisms, and the removal of debris, i.e. phagocytosis. However, neutrophils can be stimulated by a variety of means, some of which are relevant *in vivo*, and others which are merely expermental tools *in vitro*.

Stimuli which may be relevant *in vivo* include immunoglobulin, which may be in the form of immune complexes or immunoglobulin-coated particles. If the particle is too large to engulf, then stimulation will result in 'frustrated phagocytosis' (Henson and Oades 1975; Johnston and Leymeyer, 1976; Treadway et al, 1979; Gale et al, 1984). Another important group of stimuli are particles coated with C3b such as crystals (Abramson, Hoffstein and Weissman 1982; Simchowitz, Atkinson and Spilberg, 1982; Serhan et al 1984), and also the chemotactic peptide C5a (Smith and Iden, 1980). Arachidonic acid (Lew et al 1984) and leukotriene B_4 (Serhan et al, 1982 (ii); Naccache and Sha'afi, 1983) also stimulate neutrophils. *In vitro* stimuli have been used such as aggregated IgG, immunoglobulin-coated surfaces (Henson and Oades, 1975; Johnston and Leymeyer, 1976); opsonized zymosan (Roos et al, 1981); FMLP (Smith and Iden, 1980); lectins such as concanavalin A (Romeo, Zabucci and Rossi, 1973); the tumour-promoter phorbol myristate acetate (Lehrer and Cohen, 1981); lipid or water-soluble ionophores such as calcium ionophore (A23187) (Palmer and Salmon, 1983), and digitonin (Cohen and Chovaniec, 1978).

From this diverse list of stimuli, it is easy to appreciate that neutrophils can be activated without phagocytosis having to take place (Goldstein et al, 1975).

The precise details of the series of reactions which accompany cell

activation, as well as the events following activation, are dependent upon the particular stimulus used. There is considerable disagreement in the literature concerning the ability of individual agents to stimulate particular aspects of the overall activation process. Generally speaking, stimulation of neutrophils leads to aggregation, chemotaxis, phagocytosis, secretion of lysosomal enzymes, generation of oxygen-derived free radicals and release of lipid products derived from membrane phospholipids. 'Stimulus-secretion coupling' is the term coined by Weissmann and his colleagues to describe the metabolic events which occur once a cell has been activated by contact with a stimulus (Weissmann, 1982; Weissmann et al, 1983).

3.2 Events Associated with Cell Activation

Each stimulus produces a slightly different pattern of response but generally the following events occur after a ligand-receptor interaction: a change in transmembrane potential (Korchak and Weissmann, 1978; Mottola and Romeo, 1982); a loss of membrane-associated calcium at the site of interaction and a net accumulation of calcium from the extracellular medium (Naccache et al, 1979; Smolen, Korchak and Weissmann, 1981); membrane conformational changes and increase in membrane fluidity with degradation of phosphatidylinositol and formation of phosphatidic acid and other changes in membrane phospholipids (Walsh et al, 1981; Serhan et al, 1982(i); Berridge, 1984); rearrangement of microfilaments and microtubules (Roos, 1981); phosphorylation of several proteins (Roos, 1981) and generation of cyclic AMP (Herlin, Sand Petersen and Esmann, 1978). These events run in parallel and one does not necessarily lead to another.

3.2.1 Change in Transmembrane Potential

This is due to a change in the partitioning of ions across the cell membrane caused by rapid ion fluxes across the membrane. Neutrophil stimulation results in a prompt influx of sodium and calcium ions, followed by a slow potassium efflux (Romeo et al, 1975; Naccache et al, 1977; Simchowitz and Spilberg, 1979).

Whether or not an influx of sodium and the associated change in transmembrane potential is necessary for triggering neutrophil activation is a controversial point. Simchowitz and Spilberg (1979) found that FMLP-induced superoxide generation by neutrophils requires the presence of sodium ions, whereas Della Bianca et al (1983) reported that neutrophils suspended in a sodium-free, high potassium buffered solution exhibit much higher respiratory responses than those suspended in a physiologically normal medium. This however was only marked at very low concentrations of FMLP. Thus it is suggested that sodium ions regulate the threshold of responses rather than maximal responses. Other workers (Korchak and Weissmann, 1980) have also found that a reduction in sodium ion concentration depresses superoxide generation in neutrophils in response to concanavalin A and immune complexes.

3.2.2 The Role of Calcium in Cell Activation

Calcium ions have long been implicated in the initiation of neutrophil activation. At first it was considered that an influx of calcium from the extracellular medium was essential for neutrophil activation. This was based chiefly on the observation that an absence of calcium ions in the medium inhibited the release of certain mediators by particular stimuli. For example, it was found that guinea pig neutrophils failed to generate superoxide anion when stimulated with digitonin (Cohen and

Chovaniec, 1977) and that human neutrophils failed to discharge lysosomal enzymes when stimulated with opsonized zymosan (Ignarro and George, 1974) in the absence of exogenous calcium. Another observation which lent weight to this hypothesis was that calcium ions alone provoked the release of lysosomal enzymes and that calcium ionophores, which facilitate calcium influx, are in themselves stimulatory to the cells (Goldstein et al, 1974). It was also found that increasing calcium ion concentration in the medium led to progressive increases in superoxide generation whereas the introduction of a calcium channel blocker such as verapamil caused a dose-dependent inhibition of superoxide production (Simchowitz and Spilberg, 1979).

More recently however it has been shown that, although a calcium influx does take place, it is not essential. For instance, although binding of C3b to its receptor and of particulate activators of the alternative complement pathway to their receptors require calcium in the medium, binding of IgG-coated particles does not. Moreover phagocytosis of IgG-coated particles and subsequent enzyme release and respiratory burst proceed in the absence of calcium ions (Roos et al, 1981). It is now believed that it is levels of intracellular free calcium which are important. Using fluorescent probes such as chlortetracycline and Quin-2 it has been shown that membrane-bound calcium is released intracellularly upon stimulation of neutrophils with chemotactic factors (Naccache et al, 1979; White et al, 1983), with heat-aggregated IgG and Concanavalin A (Korchak et al, 1984) or with cytochalasin D and E.Coli (Takeshige et al, 1980). In addition, work with intra - and extracellular calcium antagonists has supported the hypothesis that levels of intracellular calcium are important. Stimulated neutrophils secrete lysosomal enzymes and generate superoxide anion, albeit in reduced quantities depending on the stimulus, in the presence of EGTA

(Smolen, Korchak and Weissmann, 1981). However, when the intracellular calcium antagonist TMB-8 is used, PMA-induced degranulation and superoxide generation are inhibited in a dose-dependent fashion (Smith and Iden, 1979; Smith and Iden, 1981) as are calcium ionophore-stimulated degranulation and superoxide generation (Matsumoto, Takeshige and Minakami, 1979). Thus it appears that neutrophil responses are all correlated with, and preceded by, a change in calcium permeability which results in the release of calcium from the membrane into the cytoplasm. Although an influx of extracellular calcium is not essential, it does serve to amplify the responses of the cell and different stimuli vary in their efficacy in provoking calcium permeability changes (Korchak, Rutherford and Weissmann, 1984). Mixtures of stimuli at optimal concentrations do not elicit a summation of responses, indicating that interaction of stimuli with their respective receptors regulates a common control site of calcium translocation (Korchak, Rutherford and Weissmann, 1984). It has recently been reported that a calcium efflux also occurs as a relatively late event, possibly contributing to the overall regulation of intracellular calcium levels (Korchak et al, 1984).

3.2.3 Changes in the Plasma Membrane

Perturbation of the plasma membrane, whether by attachment of phagocytic stimuli to surface receptors, or by direct interaction with the membrane results in conformational changes. There is an increase in membrane fluidity and this may increase the lateral movement of the receptor molecules in the plasma membrane which facilitates the circumferential attachment of the receptors to the opsonins on the particles (Roos, 1981). Within 15 seconds of stimulation, there are extensive changes in specific phospholipid classes (Serhan et al, 1982(i)).

Again, the precise details of which phospholipid levels rise and which decrease depends upon the stimulus, but neutrophils do rapidly 'remodel' their membranes upon activation. For instance, 5 seconds after the addition of FMLP, human neutrophil content of phosphatidylcholine, phosphatidylserine and phosphatidic acid increase, while the level of phosphatidylinositol decreases. Kinetic studies show that only phosphatidic acid levels remain elevated. In contrast, when cells are exposed to PMA, the levels of phosphatidylcholine and phosphatidylserine rapidly increase while changes in phosphatidylinositol and phosphatidic acid are not observed until after 60 seconds (Serhan et al, 1982(i)).

3.2.4 Role of Cyclic Nucleotides

Cyclic AMP and agents that raise intracellular levels of cyclic AMP inhibit degranulation, whereas cyclic GMP and agents that elevate levels of cyclic GMP have the opposite effect (Ignarro and Cech, 1976). It was originally thought that there was no change in the levels of intracellular cyclic AMP upon neutrophil stimulation and that it was cyclic GMP levels that rose (Ignarro and George, 1974). However, the assays used were not sensitive enough as Herlin et al (1978) demonstrated a rise in cyclic AMP levels, 15 seconds after stimulation with latex particles, with levels returning to normal after 1 to 2 minutes. Again, however, the precise details of cyclic AMP kinetics depend upon the stimulus (Smolen, Korchak and Weissmann, 1980), with some agents able to stimulate degranulation and oxidative metabolism without causing a rise in cyclic AMP.

4. THE CONSEQUENCES OF CELL ACTIVATION

The events which may occur as a direct consequence of cell activation are aggregation and adherence, chemotaxis, degranulation, oxidative metabolism and arachidonic acid metabolism (Figure 1.1). Aggregation and adherence are briefly mentioned first; chemotaxis has been discussed in section 1.3. The biochemical details of degranulation and oxygen-derived free radical production are then discussed before being put into the context of their function *in vivo*, i.e. bacterial killing. The relevance of the products of oxidative metabolism and arachidonic acid metabolism in mediating tissue damage is then discussed in section 7.

Following activation, neutrophils aggregate and adhere to surfaces. Dahinden and colleagues have shown that cell surface contact plays an essential role in triggering the respiratory burst of neutrophils activated by soluble stimuli (Dahinden, Fehr and Hugli, 1983), and that, for aggregation to occur, the presence of oxygen metabolites and degranulation products are not necessary (Kaplan et al, 1982). Following activation,neutrophils also become sensitive to chemotactic gradients and exhibit chemotaxis, as already described.

4.1 Degranulation

Following interaction of the neutrophils with the inflammatory stimulus, the membranes of the lysosomal granules fuse with the plasma membrane and the contents of the granules are discharged. If the stimulus has been phagocytosed the granule contents are released into the newly formed phagocytic vacuole or phagosome (Stossel, 1976). In the neutrophil, the granules can be divided into 2 categories. The first group, analagous with lysosomes of other tissues,comprise the azurophil, or primary granules, which are large and relatively dense. They

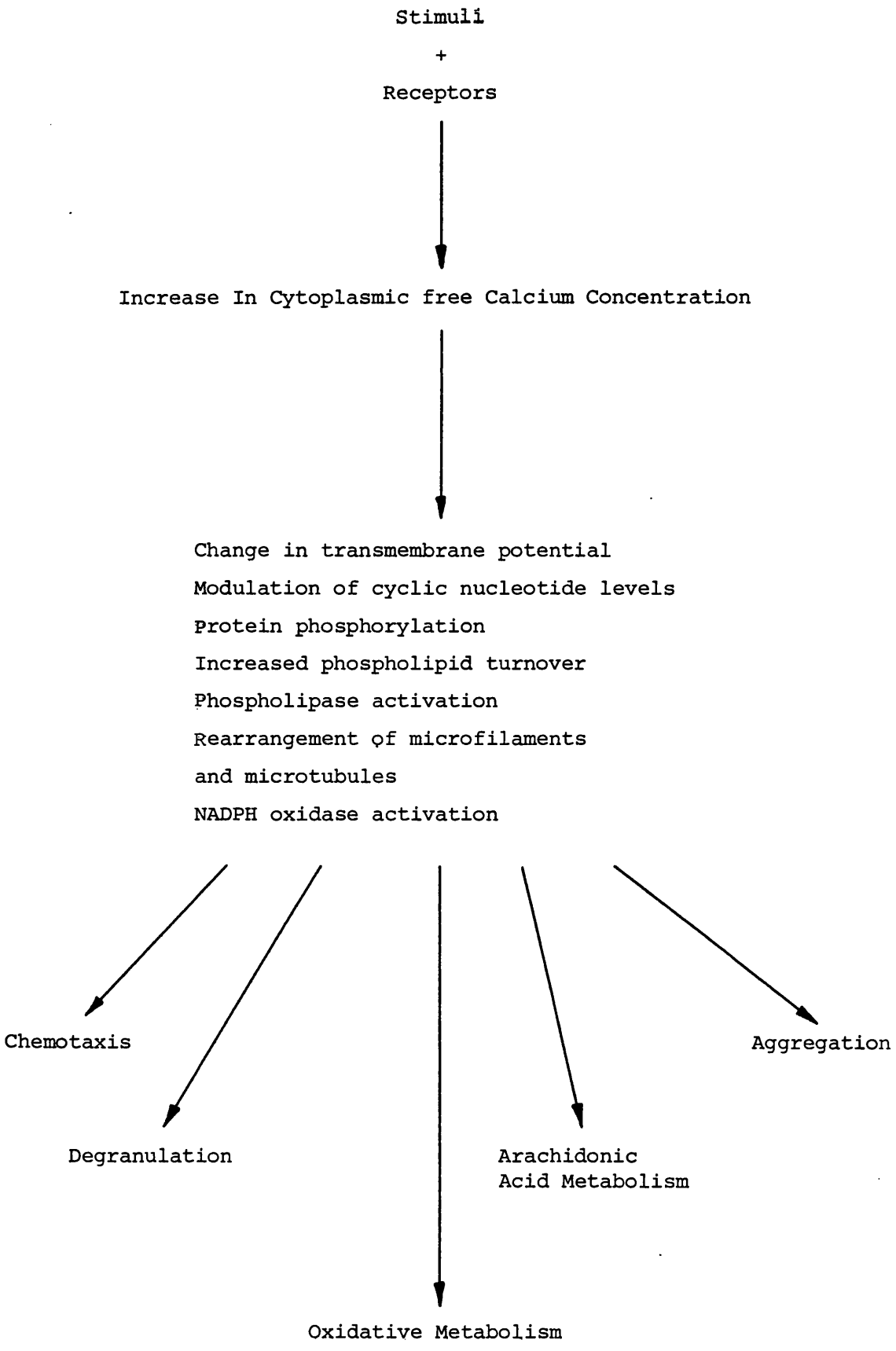


Figure 1.1 Events concurrent with Neutrophil Activation

are formed during the promyelocyte stage of development. They contain the acid hydrolases, myeloperoxidase and lysozyme. A sub-class of these granules, which are morphologically more heterogeneous and are of low density, also contain acid hydrolases, but little or no myeloperoxidase. These form 10% to 20% of all the granules. The second group, formed during the myelocyte stage of development, are the specific, or secondary granules. These are smaller and less dense and contain lactoferrin, alkaline phosphatase and some lysozyme. These form 80% to 90% of all the granules (Elsbach, 1974; Baggiolini, 1982). Both azurophil and specific granules participate in degranulation in response to phagocytosis. The specific granules fuse within 3 to 5 minutes, whereas the azurophil granules fuse after 5 to 10 minutes (Hirsch, 1974).

Activation of the plasma membrane by soluble stimuli also causes the release of granule contents (Smith and Iden, 1980). This appears to affect mainly the specific granules rather than the azurophil granules, with the release of large amounts of lactoferrin and lysozyme (Segal, 1981). Phorbol myristate acetate has been shown to stimulate the release of the contents of specific, rather than azurophil granules (Lisiewicz, 1980). As is the case with the generation of oxygen metabolites, calcium has been shown to be important in the process of degranulation. Phorbol myristate acetate-induced release of lysozyme is inhibited by the intracellular calcium antagonist, TMB-8 (Smith and Iden, 1979), whereas the release of β-glucuronidase and lysozyme in response to FMLP, calcium ionophore, concanavalin A, opsonized zymosan, heat-aggregated IgG and phorbol myristate acetate proceeds normally in the presence of EGTA (Smolen, Korchak and Weissmann, 1981). Calcium ions, in the absence of other stimuli, are capable of provoking the release of lysozyme from neutrophils in a concentration, time and temperature-

dependent fashion (Goldstein et al, 1974).

Intracellular cyclic GMP enhances the release of granule enzymes, whereas intracellular cyclic AMP inhibits the process (Ignarro and George, 1974; Ignarro and Cech, 1976).

Anion transport may also be involved in degranulation because blocking of anion efflux inhibits the process (Korchak et al, 1980). As degranulation can be restored by the addition of calcium, an explanation could be that the calcium liberated from the membrane pairs with the anions, thus decreasing the concentration of free intracellular calcium (Roos, 1981).

4.2 Oxidative Metabolism - The Respiratory Burst

Following activation there is a change in oxygen metabolism by neutrophils. There is a sharp increase in oxygen uptake, sometimes more than 50-fold (Babior, 1978); the production of superoxide anion and hydrogen peroxide (Babior, Kipnes and Curnette, 1973; Root et al, 1975) and the increased metabolism of glucose via the hexose monophosphate (HMP) shunt (Nelson et al, 1980).

Although this series of changes is known as the respiratory burst, the increase in oxygen uptake is insensitive to inhibitors of mitochondrial respiration and hence cannot be explained in terms of increased oxygen required to provide ATP as a source of energy for phagocytosis as originally thought (Sbarra and Karnovsky, 1959). Rather, all the oxygen is utilized to produce superoxide anion ($O_2^{-\bullet}$) (Weening, Wever and Roos, 1975), via a one-electron reduction using NADPH as the electron donor. Superoxide is an oxygen molecule with an extra, unpaired electron in an outer orbital:

$$2O_2 + NADPH \xrightarrow[\text{oxidase}]{\text{NADPH}} 2O_2^{-\bullet} + NADP^+ + H^+$$

Superoxide is either released into the extracellular environment, or it may remain associated with the oxidase system (Segal and Meshulam, 1979).

Most of the superoxide (80%) is converted to hydrogen peroxide by dismutation, and this accounts for practically all the hydrogen peroxide formed during the respiratory burst (Root and Metcalf, 1977):

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

This dismutation can be spontaneous (especially in acid conditions) or accelerated by the ubiquitous cytoplasmic enzyme superoxide dismutase (SOD) (Baehner et al, 1975; Fridovich, 1978). At the same time, glucose is metabolised through the HMP shunt in order to regenerate the NADPH that has been consumed both by the $O_2^{\bullet-}$-forming enzyme, and by a glutathione-dependent hydrogen peroxide detoxifying system that is found in the cytoplasm of the neutrophil. This system disposes of hydrogen peroxide by using it to oxidise glutathione in a reaction catalysed by glutathione peroxidase:

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

The oxidised glutathione is then reconverted to reduced glutathione by the glutathione reductase reaction in which $NADP^+$ is produced (Weiss and LoBuglio, 1982):

$$GSSG + NADPH \longrightarrow 2GSH + NADP^+$$

4.2.1 The Oxidase Enzyme

The nature and location of the enzyme that catalyses the reduction of oxygen to produce superoxide has been the subject of innumerable papers in the literature over the last decade. Several oxidases have been

suggested as responsible (DeChatelet, 1978; Rossi et al, 1982), the main two contenders being membrane-bound NADH oxidase and membrane-bound NADPH oxidase. There have been a number of approaches attempting to isolate and characterize the respiratory enzyme. Several different species were employed; resting cells were used by some investigators, but stimulated cells by others; and "one enzyme - one substrate" was assumed (Badwey, Curnette and Karnovsky, 1979). By using subcellular fractions from neutrophil homogenates some laboratories put forward the theory that NADH oxidase produced $O_2^{\bullet-}$ in the presence of NADH as substrate (Baehner, Gilman and Karnovsky, 1970; Johnston et al, 1975), but the majority view is that NADPH oxidase is the enzyme responsible (McPhail, DeChatelet and Shirley, 1976; Nakamura, Baxter and Masters, 1981; McPhail and Snyderman, 1983). It appears to be an FAD-requiring enzyme (Babior and Kipnes, 1977) which utilizes NADPH as the substrate but it is also capable of using NADH, albeit less efficiently (Babior, Curnette and McMurrich, 1976). It is also hypothesized that the oxidase is a multicomponent electron transport system and contains a cytochrome as a second electron carrier. There is a b-type cytochrome, found uniquely in phagocytes, which is associated with the respiratory burst oxidase (Segal and Jones, 1978, 1979) and it has been shown to be capable of transferring electrons from NADPH to oxygen, although whether it is actually on the electron transport path between NADPH and oxygen, or whether it plays a role in converting the oxidase from its resting to its activated state remains to be elucidated (Gabig, Schervish and Santinga, 1982). Also, a quinone, ubiquinone-10, has been found associated with the enzyme system and its participation in the initiation of the respiratory burst has been postulated (Cunningham et al, 1982).

The enzyme has long been thought to be associated with the cell surface (Goldstein et al, 1977) and is now considered to be embedded in the plasma membrane with the NADPH binding site projecting into the cytosol and the rest in the lipid bilayer (Dewald et al, 1979; Babior et al, 1981). What actually activates the enzyme and hence initiates the respiratory burst is poorly understood. When the isolated particulate fractions containing the enzyme are treated with p-diazobenzenesulphonic acid, and then stimulated with FMLP or concanavalin A, $O_2^{\bullet -}$ production is inhibited, but not if phorbol myristate acetate or calcium ionophore are the stimulus. Thus it appears that more than one mechanism exists for the activation of the oxidase (McPhail, Henson and Johnston Jr, 1981). This is supported by the observation that, depending upon the stimulus, activation is or is not associated with a lag period (McPhail and Snyderman, 1983) and by the observations already mentioned that it does or does not require a calcium influx; a change in transmembrane potential; increments in cyclic AMP or breakdown of phosphatidylinositol. The current hypothesis seems to be that the activation of the oxidase might be the consequence of a change in the state of phosphorylation of an activator protein (Babior, 1984) via protein kinase C. This protein kinase C, which is calcium/phosphatidylserine/diacylglycerol dependent (De Riemer et al, 1985), is either activated by the increase in intracellular calcium concentration or by phosphatidylinositol breakdown (Rink, Sanchez and Hallam, 1983). The only stimulus not provoking an increase in cytosolic calcium is phorbol myristate acetate, but this has been shown to directly activate the protein kinase C itself (Niedel, Kuhn and Vandenbark, 1983; De Riemer et al, 1985). It has been shown that the enzyme system does require calcium ions for full expression of catalytic activity (Green, Wu and Wirtz, 1983).

In addition, it has been reported that exposure of neutrophils to FMLP, before using another stimulus, potentiates the activation of the respiratory burst enzyme (Bender, McPhail and Van Epps, 1983) and it is FMLP which provokes the greatest increase in calcium permeability (Korchak, Rutherford and Weissman, 1984).

Addition of the calcium-binding protein, calmodulin to preparations of NADPH oxidase stimulates enzymatic rates and addition of calmodulin and calcium,but not calcium alone, to preparations of NADPH oxidase inactivated by EDTA results in restoration of activity. This suggests a role for calmodulin in the control of the oxidase but not in its activation (Jones et al, 1982).

4.2.2 Abnormalities of the Oxidase

There is a complete absence of a respiratory burst associated with the neutrophils from patients suffering from the inherited syndrome chronic granulomatous disease (CGD) (Johnston et al, 1975; Briggs, Karnovsky and Karnovsky, 1977; Segal, 1981). This is considered to be due to a defect in the oxidase enzyme and its activity is greatly reduced in particles from stimulated neutrophils from patients with CGD (Babior, Curnette and McMurrich, 1976; Babior, 1978). Furthermore it has been demonstrated that there is an absence or a gross abnormality of the cytochrome b in patients with the disease (Segal et al, 1978). The implication of this abnormality will be discussed in section 4.2.5 when the role of free radical production and degranulation *in vivo* is discussed.

4.2.3 Formation of Hydroxyl Radical

Haber and Weiss proposed in 1934 that a hydroxyl radical could be formed

from the reduction of H_2O_2 by $O_2^{\bullet -}$:

$$O_2^{\bullet -} + H_2O_2 \longrightarrow OH\bullet + OH^- + O_2$$

Beauchamp and Fridovich in 1970, were the first to demonstrate that OH• could indeed be generated from a reaction between $O_2^{\bullet -}$ and H_2O_2, using the enzymatic system xanthine/xanthine oxidase. However, it was concluded that this precise reaction could not produce physiologically relevant amounts of OH• because the rate constant for the interaction of $O_2^{\bullet -}$ with H_2O_2 is substantially less than that of the competing reaction, namely the spontaneous dismutation of $O_2^{\bullet -}$. Thus although representing the overall stoichiometry of the process, it cannot be the reaction mechanism. McCord and Day (1978) proposed a modification of the Haber-Weiss reaction in which iron serves as a redox catalyst, and the overall reaction is the sum of these reactions:

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$\underline{Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\bullet}$$

$$O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + OH^- + OH\bullet$$

The feasibility of this reaction has since been confirmed by others (Halliwell, 1978).

There is considerable argument in the literature concerning the relevance of this reaction <u>in vivo</u>, and whether there is sufficient free iron in extracellular fluids to catalyse the reaction, for unless it is present in its 2 valence states, it is unable to participate in this reaction. Thus iron as part of cytochromes, haemaglobin and catalase would appear unable to catalyse the reaction (Gutteridge, 1982). Although iron in animal tissues is largely bound to enzymes or to proteins such as transferrin or ferritin, iron ions are moving between

their storage/transport sites and their sites of use and some of them should become attached to the phosphate groups of membrane lipids. Biological phosphate esters (e.g. ATP and GTP) can also remove iron from protein complexes (Halliwell, 1982). Also micromolar concentrations of non-protein bound iron has been detected in human synovial and cerebrospinal fluids (Gutteridge et al, 1982; Rowley et al, 1984). Several groups have now reported that OH• can be generated either by using isolated NADPH oxidase, utilizing transferrin as the catalyst (Bannister et al, 1982) or by using hypoxanthine / xanthine oxidase enzymatic system, utilizing transferrin as catalyst (Motohashi and Mori, 1983). Other groups have successfully used lactoferrin as the catalyst (Ambruso and Johnston Jr., 1981). However, the relevance of these experiments may be questioned because they used iron-binding proteins fully saturated with iron, which seldom occur in vivo. Indeed, other groups have been unable to show that protein-bound iron does catalyse OH• formation (Winterbourn, 1982). Most recently it has been reported that superoxide generated by stimulated neutrophils can mobilize free iron from ferritin and thus make it available as a catalyst (Biemond et al, 1984). This is especially interesting in the light of the finding that rheumatoid synovial fluid contains 9 times higher concentration of ferritin than normal synovial fluid (Blake et al, 1980). Another argument against iron-catalysed generation of OH• being relevant in vivo is the fact that the concentration of superoxide present will be much less than that of other reducing agents such as glutathione and ascorbic acid which are potentially capable of reducing Fe^{3+} (Winterbourn, 1982). However in vitro it has been shown that glutathione does not prevent superoxide and hydrogen peroxide-dependent formation of OH• in the presence of iron salts (Rowley and Halliwell, 1982) and that ascorbic acid reacts so rapidly with hydrogen peroxide,

superoxide and OH• that it is quickly destroyed (Rowley and Halliwell, 1983). This might explain the low ascorbate concentrations found in synovial fluid of rheumatoid patients (Blake et al, 1981(i)). The relevance of iron-catalysed OH• production to tissue damage will be discussed later in section 7.1.2 of this Introduction. The generation of oxygen metabolites by stimulated neutrophils is summarised in Figure 1.2.

4.2.4 Singlet Oxygen

The outer electrons of oxygen can change their spin and occupy the same or different orbitals upon absorption of energy (Badwey and Karnovsky, 1980). Two such excited states of oxygen can be formed and these are termed singlet oxygen, $^1O_2$.

When the two outer electrons occupy the same orbital and are of opposite spin this is designated delta singlet oxygen, $^1\Delta gO_2$, and when the two outer electrons still occupy separate orbitals, but are of opposite spin this is designated sigma singlet oxygen, $^1\Sigma^+gO_2$. $^1\Sigma^+gO_2$ has a higher energy than $^1\Delta gO_2$ and is thus more unstable, having a lifetime in solution of 10^{-11} seconds whereas $^1\Delta gO_2$ has a lifetime of 2×10^{-6} seconds (Foote, 1976).

A number of reactions are capable of generating $^1O_2$ under conditions which may be relevant <u>in vivo</u> :

$$HO_2^{\bullet} + O_2^{-\bullet} + H^+ \longrightarrow {}^1O_2 + H_2O_2$$

$$O_2^{-\bullet} + H_2O_2 \xrightarrow{\text{metal}} {}^1O_2 + OH^{-\bullet} + OH^{\bullet}$$

$$OCl^{-\bullet} + H_2O_2 \longrightarrow {}^1O_2 + Cl^- + H_2O$$

$$O_2^{-\bullet} + OH^{\bullet} \longrightarrow {}^1O_2 + OH^{-\bullet}$$

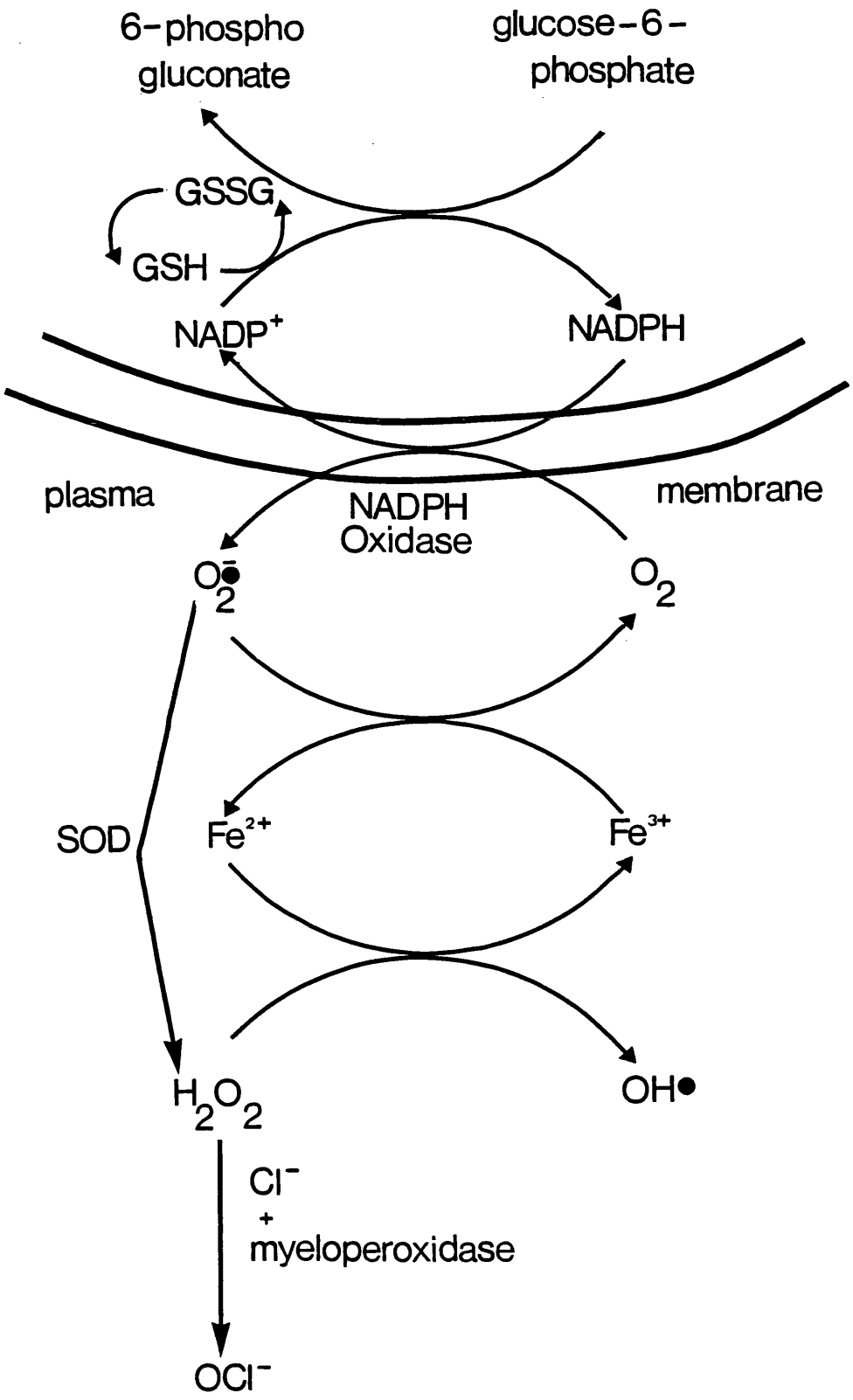


Figure 1.2 Generation of oxygen metabolites by stimulated neutrophils.

Differences in Oxidative Metabolism Between Neutrophils and Mononuclear Phagocytes

Monocytes also show an increase in oxygen consumption upon stimulation, together with production of $O_2^{\bar{\bullet}}$ and H_2O_2 (Sagone Jr., King and Metz, 1976). However, it has been suggested in comparative studies that neutrophils consume 2½ times as much oxygen, generate twice as much $O_2^{\bar{\bullet}}$ and release 5 times as much H_2O_2 as monocytes in response to a phagocytic stimulus (Reiss and Roos, 1978). Furthermore, Antimycin A, a specific mitochondrial respiratory chain inhibitor, inhibits oxygen consumption in monocytes by 70% which would imply that only 30% of the oxygen consumed by activated monocytes is converted to H_2O_2 and 70% is metabolized by the mitochondrial respiratory chain. In studies using phorbol myristate acetate and micropore filters coated with aggregated IgG, the release of $O_2^{\bar{\bullet}}$ from monocytes was about two-thirds that from neutrophils from the same donor (Johnston, Lehmeyer and Guthrie, 1976). There is some evidence to suggest that macrophages from mouse bone marrow may use NADH oxidase as the respiratory enzyme rather than NADPH oxidase (Hovestadt and Ferber, 1984).

4.2.5 Microbicidal Killing

Whilst phagocytosis ensures the sequestration of potentially harmful microorganisms, they still have to be killed to prevent replication and consequent spread of infection, and it is the purpose of the respiratory burst to provide a battery of oxidizing agents that can be used by the phagocyte for the destruction of microorganisms. Patients with chronic granulomatous disease, where there is an absence of any respiratory burst, suffer a high incidence of recurrent infection, which underlines the importance of reactive oxygen metabolites in microbicidal killing (Segal, 1981).

Superoxide itself is probably not bactericidal as it is only weakly reactive (Badwey and Karnovsky, 1980) and has a half-life, in the presence of SOD, of only 5 milliseconds (Greenwald, 1981). It is H_2O_2 which is important in bacterial killing. It has itself a certain amount of bactericidal potency, but this is greatly augmented through the action of the enzyme myeloperoxidase (Klebanoff, 1967; Rosen and Klebanoff, 1979). This haemoprotein is present in the azurophilic granules of neutrophils and monocytes (Babior, 1978). It catalyzes the oxidation of halide ions to hypothalite ions by H_2O_2.

$$X^{-\bullet} + H_2O_2 \longrightarrow XO^{-\bullet} + H_2O$$

Cl^-, Br^- and I^- can all be oxidized by this enzyme, but it is likely that Cl^- is the physiologic substrate, since it is by far the most abundant halide in the cell, being present in neutrophils at a concentration of approximately 90mM (Badwey and Karnovsky, 1980). The mechanism by which hypochlorite is bactericidal may be due to halogenation of the bacterial cell wall. Hypochlorite is itself an exceedingly powerful microbicidal agent and it is also the precursor of the choramines, a group of microbicidal oxidized halogens that are formed by the reaction between hypochlorite and ammonia, or amines (Babior, 1984):

$$OCl^{-\bullet} + RNH_3 \longrightarrow RNH_2Cl + OH^{-\bullet}.$$

The myeloperoxidase system is also able to decarboxylate amino acids, converting them to aldehydes, carbon dioxide and ammonia :

$$R\text{-}CHNH_2 - COOH \longrightarrow R\text{-}CHO + CO_2 + NH_3$$

The aldehydes thus formed may be toxic. Recently it has been suggested that myeloperoxidase in this system can be replaced by Fe^{2+} and the chloride ions with iodide ions (Klebanoff, 1982). It is proposed that

Fe^{2+} and H_2O_2 generate $OH^{\bullet}$, which interacts with iodide to form one or more toxic species, as well as being a potential microbicidal agent itself. This mechanism could be important in patients with myeloperoxidase deficiency, many of which are in fact asymptomatic (Segal, 1981). Myeloperoxidase-deficient cells show an enhanced respiratory burst when compared with normal cells, which supports this theory (Dri et al, 1984).

The killed organisms are subsequently digested and degraded by the proteolytic enzymes released upon degranulation. Lysozyme attacks the mucopeptide of cell walls of some species (Stossel, 1976). The phagocytic vacuoles start to swell, resulting from the attraction of water into the vacuoles by osmosis. As the vacuoles swell they fuse with each other and the plasma membrane and then exocytose the partially degraded organisms and digestion products to the exterior (Segal, 1981).

4.3 Arachidonic Acid Metabolism

The recognition that the plasma membrane is the site of reception, modulation and transfer of signals that lead to the cell's responses has aroused much interest in the possible role of membrane phospholipids in trans-membrane signalling.

The neutrophil is an example of a cell thought to undergo phospholipid modification upon stimulation with deacylation and reacylation and a liberation of unsaturated fatty acids (Stocker and Richter, 1982). The 20-carbon polyunsaturated fatty acid arachidonic acid is released from membrane phospholipid, particularly phosphatidylinositol and phosphatidylcholine (Walsh et al, 1981). Under resting conditions the cells contain little if any free arachidonic acid (Naccache and Sha'afi, 1983). It is released from its esterified form by phospholipase A_2

(Walsh et al, 1983). This calcium - and calmodulin-dependent enzyme (Stocker and Richter, 1982) has been shown to be associated with the membrane of human neutrophils (Franson et al, 1977; Victor et al, 1981) and thus is present in a strategic location at the cell surface. Phospholipase A_2 releases arachidonic acid and lysophospholipids (Englberger, Bitter-Suermann and Hadding, 1984). Free arachidonic acid is rapidly converted to sets of biologically active compounds - prostaglandins, thromboxanes and leukotrienes. The synthesis of prostaglandins and the thromboxanes is initiated by 11-cyclooxygenase and the synthesis of the leukotrienes by the iron-containing dioxygenases, the lipoxygenases (Taylor and Morris, 1983). In neutrophils, the metabolism of arachidonic acid proceeds predominantly through the 5-lipoxygenase pathway (Naccache and Sha'afi, 1983). Recently a 15-lipoxygenase has also been described (Samuelsson, 1983) and several other lipoxygenases with differing positional specificities have been identified in other cell types. Figure 1.3 illustrates the major pathways of arachidonic acid metabolism.

4.3.1 The 11-Cyclooxygenase Pathway

The initial step in the metabolism of arachidonic acid by 11-cyclooxygenase is the addition of molecular oxygen to give the endoperoxide PGG_2. This is rapidly transformed to the hydroxyendoperoxide PGH_2, with the liberation of an oxygen-derived free radical. Once the endoperoxides have formed, they have a very short half-life in cells and fluids and they become transformed into prostaglandins, prostacyclin and thromboxane (Weissmann, 1983; Bakhle, 1983).

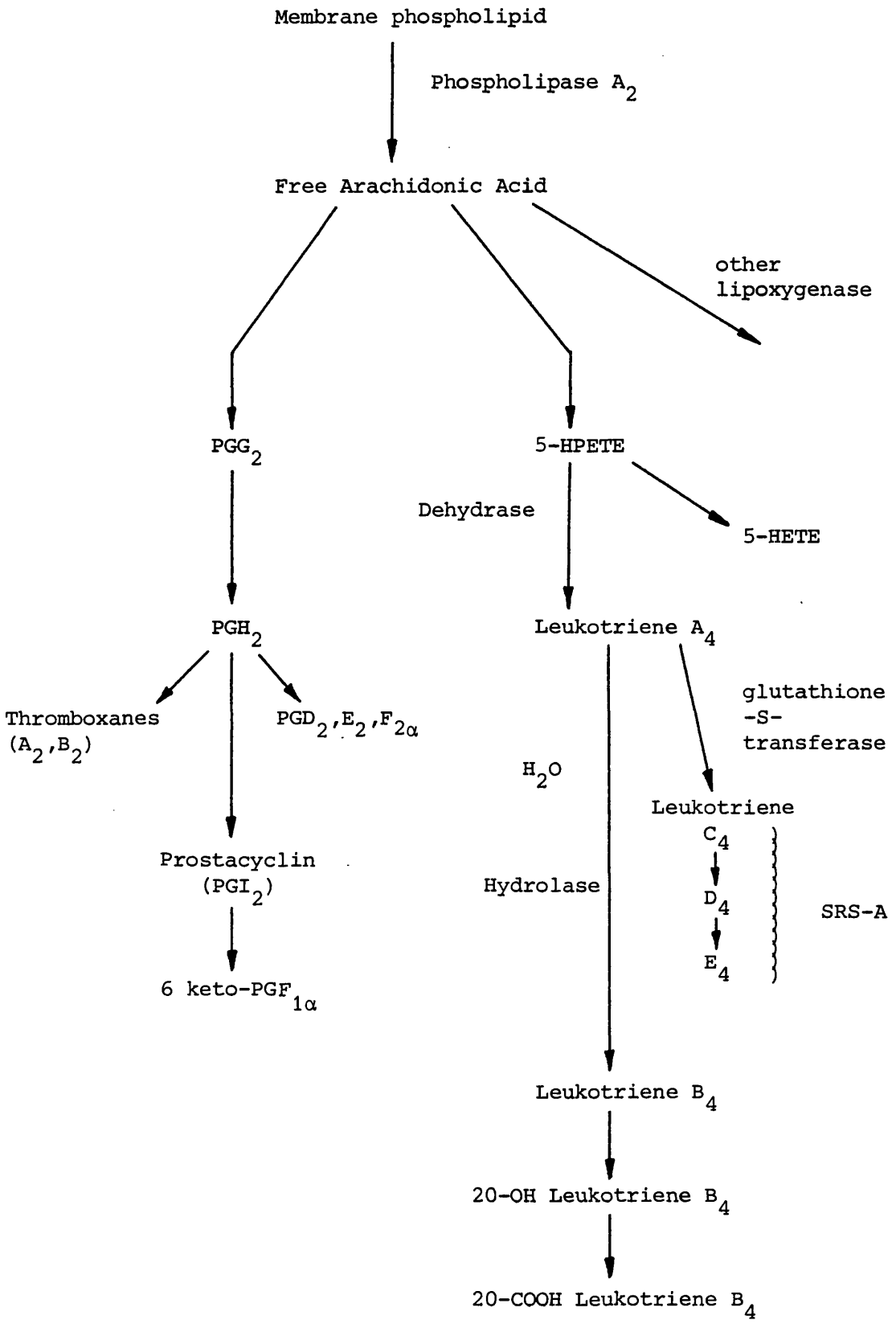


Figure 1.3 Metabolism of Arachidonic Acid

4.3.2 The 5-Lipoxygenase Pathway

The initial step in the metabolism of arachidonic acid by 5-lipoxygenase is the formation of the 5-hydroperoxy derivative. This can either be spontaneously hydrolysed to form the 5-hydroxyeicosatetraenoic acid (5-HETE) or be enzymatically converted to an unstable epoxide, leukotriene A_4, that reacts rapidly with water to form leukotriene B_4, a dihydroxy-derivative of arachidonic acid. In the presence of glutathione, leukotriene A_4 is converted to leukotrienes C_4, D_4 and E_4. Leukotriene B_4 has been shown to be W-oxidized to form 20-OH leukotriene B_4 which can be further metabolized to the dicarboxylic acid 20-COOH-leukotriene B_4 (Piper, 1984).

The structures of the metabolites of the 5-lipoxygenase pathway are given in Figure 1.4. Leukotriene B_4 thus formed is now capable of stimulating neutrophils themselves and thus a perpetuating cycle of activation may well be set up.

Leukotriene B_4 is a potent and multifunctional neutrophil stimulus (Naccache and Sha'afi, 1983). It stimulates chemotaxis and aggregation (Prescott, Zimmerman and Seeger, 1984). It can also stimulate degranulation, oxygen uptake and superoxide generation, dependent on calcium in the medium (Sumimoto, Takeshige and Minakami, 1984) although it is not as potent as FMLP (Palmblad et al, 1984). The fact that an inhibitor of leukotriene synthesis, U-60 257 also inhibits degranulation and superoxide generation suggests that LTB_4 might function to mediate neutrophil activation (Smith et al, 1982). This activity is probably related to its ability to mobilize calcium by essentially the same mechanisms as chemotactic factors such as FMLP i.e. it causes a rapid rise in intracellular calcium due to both an extracellular influx and release from intracellular pools (Naccache et al, 1984; Lew et al, 1984; Becker et al, 1981),

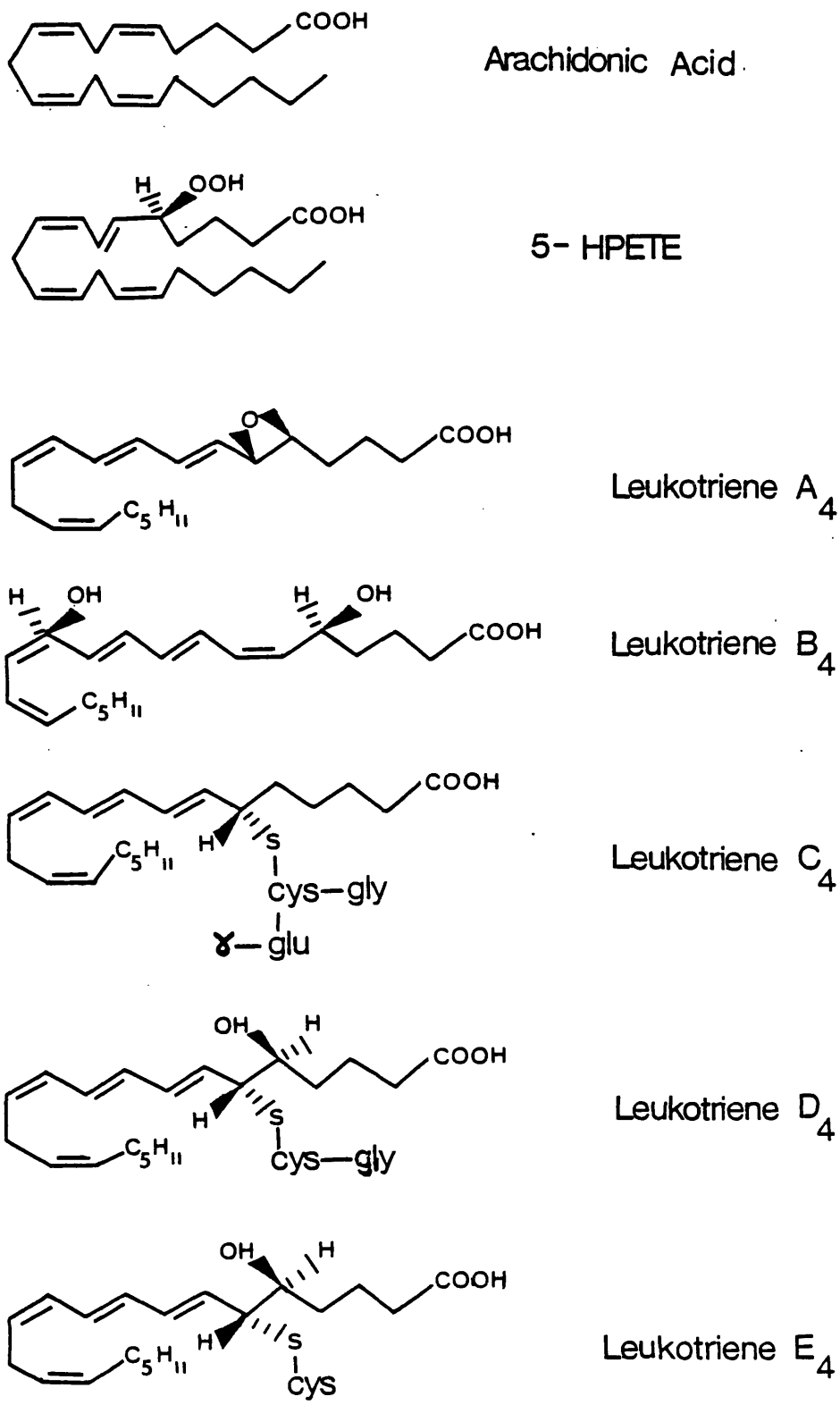


Figure 1.4 Products of the 5-lipoxygenase pathway derived from arachidonic acid.

Whereas the products of the lipoxygenase pathway, especially LTB_4, are capable of stimulating further neutrophil responses, the products of the cyclooxygenase pathway are not. Prostaglandins of the E series and prostacyclin all inhibit superoxide production and LTB_4 production (Fantone and Kipnes, 1983; Ham et al, 1983). This may well be linked with the fact that these prostaglandins raise levels of cyclic AMP within cells.

5. IMPORTANCE OF FREE SULPHYDRYL GROUPS FOR NEUTROPHIL FUNCTION

Both penetrating sulphydryl group inhibitors such as mercuric chloride and N-ethylmaleimide, and surface sulphydryl group inhibitors such as para-hydroxymercuriphenylsulphonic acid (pHMPSA) have been used to discover the role of sulphydryl groups in various neutrophil functions. Penetrating sulphydryl group inhibitors have been found to inhibit neutrophil adhesiveness, motility, phagocytosis and superoxide production (Giordano and Lichtman, 1973; Tsan, Newman and McIntyre, 1976; Cohen and Chovaniec, 1978). Surface sulphydryl group blockers inhibit HMP shunt activity (Tsan et al, 1976) but their reported effects on oxidative metabolism is conflicting. Giordano and Lichtman claim that pHMPSA has no effect on any neutrophil function, whereas Tsan, Newman and McIntyre claim that whilst pHMPSA has no effect on superoxide production, it does inhibit H_2O_2 production.

There has been interest in the role of serine proteases (or esterases) on neutrophil and mononuclear phagocyte functions. Using potent inhibitors or synthetic substrates of serine esterases, several studies have shown that protease activity may be essential for superoxide production (Kitagwa, Takaku and Sakamoto, 1980; Hoffman and Autor, 1982) and it has been suggested that the protease involved in the production

of $O_2^{\bullet -}$ is a chymotrypsin - or trypsin-like serine esterase. These inhibitors are potent sulphydryl group inhibitors and it is now suggested that their effects may be due to this fact (Kitagwa, Takaku and Sakamoto, 1980). This is further substantiated by the fact that inhibition of $O_2^{\bullet -}$ production by neutrophils by these inhibitors can be prevented by GSH (Tsan, 1983).

6. ROLE OF NEUTROPHILS IN MEDIATING THE INFLAMMATORY PROCESS

It is important to appreciate that acute inflammation is essentially a curative process for damaged tissue. Sometimes, however, instead of resulting in healing, inflammation becomes chronic, resulting in gradual destruction of tissue.

When tissue is injured, a host of inflammatory mediators from mast cells, disruption of blood vessels and damage to cells are released. These mediators cause vasodilation and increased vascular permeability leading to vascular leakage. Such mediators from plasma include kinins (e.g. bradykinin) and C5a. Those released from tissues include histamine, 5-hydroxytryptamine (5-HT), LTB_4 and prostaglandins (Ryan and Majno, 1977). It has also been suggested that neutrophils, responding to chemical signals in the tissue such as LTB_4 or C5a can control the permeability of the blood vessel wall to plasma proteins (Wedmore and Williams, 1981). The vascular leakage results in swelling which feels hot, painful and is associated with redness, and is due to leakage of plasma into the tissues. Further factors in this plasma which perpetuate the inflammatory process include complement components and components of the clotting system. All these mediators will attract neutrophils and recruit them to the site of the lesion. In fact, the neutrophil is the first cell to appear in the

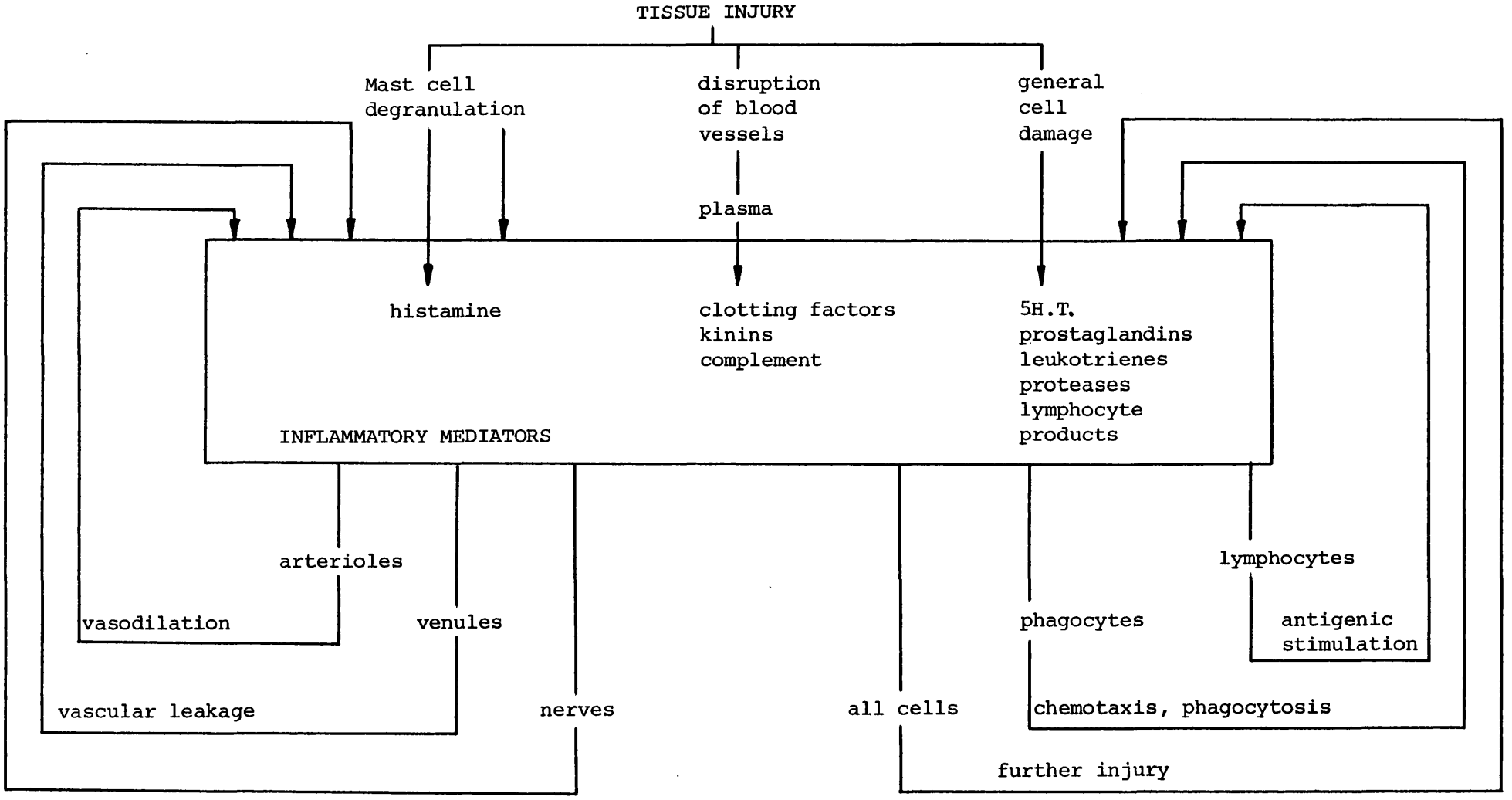


Figure 1.5 Summary of the principal mechanisms of the Inflammatory Process

acute inflammatory exudate. Circulating neutrophils exhibit increased adherence to endothelial cells in the vicinity of the lesion. This enhanced stickiness of the endothelium appears to be a prerequisite for their subsequent emigration into the tissues. They penetrate the vessel wall and squeeze between the endothelial cells (diapedesis) into the surrounding tissues. This takes between 2 and 12 minutes. Once they have arrived at the place where the concentration of the chemotactic factors is high, neutrophils respond by significant release of granule enzymes, oxygen metabolites and leukotrienes, all further mediators of inflammation. High concentrations of chemoattractants tend to reduce the motility of the cells and hence increasing numbers of neutrophils tend to become immobilized in such an environment (Zigmond, 1978). Stimulated neutrophils have been shown to induce the release of prostacyclin from cultured endothelial cells (Harlan and Callahan, 1984). Prostacyclin has a pro-inflammatory action and enhances oedema formation due to the vasodilation it causes. Cells from CGD patients do not have this effect and the presence of catalase significantly inhibits prostacyclin release, which implies that H_2O_2 generation is responsible. As quinacrine inhibits the release of prostacyclin, it is suggested that H_2O_2 acts by triggering endothelial cell membrane phospholipase.

Thus, inflammation is a self-perpetuating process, and continues until the stimulus is removed. This "vicious circle" of events is shown in Figure 1.5.

7. THE ROLE OF NEUTROPHILS IN MEDIATING TISSUE DAMAGE IN RHEUMATIC DISEASES

7.1 Rheumatoid Arthritis

Rheumatoid arthritis is an example of a disease where there appears to

be insufficient control over the inflammatory process. It is a systemic disease of connective tissue which affects mainly the peripheral synovial joints. Symmetrical joints are affected and are most commonly the proximal, interphalangeal and metacarpophalangeal joints, wrists, metatarsophalangeal joints and the knees. Age of onset is usually between 25 to 55 and 3% of women and 1% of men in the total population are affected. There may be a family predisposition to the disease and its severity is associated with an increased frequency of the HLA antigen DR4. In addition, those having the DR3 antigen have a more aggressive disease (Holland and Jayson, 1981). The cause of RA is unknown. It is thought that, in a genetically predisposed individual, some antigenic environmental trigger stimulates the production of auto-antibodies. Tissue damage results from immune complex deposition in the synovium and blood vessel walls followed by activation of complement and release of inflammatory mediators such as enzymes and oxygen-derived free radicals from phagocytic cells (Zvaifler, 1971; Movat, 1979). During episodes of active arthritis the joints are painful and swollen with loss of movement and function. This is most apparent on waking and gives rise to 'early morning stiffness'. These features are due to synovial inflammation, oedema and hypertrophy and the development of joint effusions. As the patient finds the most comfortable position is for the inflamed joint to be flexed, fixed flexion deformities can arise and weakening of the joint capsule and tendon along with ligament damage leads to instability and subluxation. Eventually severe joint damage may lead to fibrous or bony ankylosis or secondary degenerative changes as the arthritis becomes chronic (Holland and Jayson, 1981).

Other features of the disease include rheumatoid nodules, which are granulomatous masses found at pressure areas. Their presence indicates

severe disease. Common systemic symptoms of the disease include weight loss and anaemia (Panayi, 1980).

7.1.1 The Synovium

When the synovium becomes inflamed, finger-like villi are formed in the synovial tissues. Synovial cells undergo hyperplasia, accompanied by cell hypertrophy and multinucleate giant cell formation. There is infiltration of the subsynovial tissue by monocytes, plasma cells and small lymphocytes (Vernon-Roberts, 1980). Rheumatoid synovial cells show increased PGE_2 and collagenase production (McGuire et al, 1982; Baker et al, 1983) due to the action of a monocyte-derived factor which is probably related to Interleukin I . As inflammation proceeds the inflamed synovial villi proliferate and spread over the joint surface as pannus. This progresses from the joint periphery towards its centre, eroding cartilage and bone along its path. The pannus may then become fibrous, restricting movement of the joint (Figure 1.6). Active disease is accompanied by an increase in the amount of synovial fluid. Normally in the knee, only about 2ml is present which is clear, pale yellow, highly viscous and contains few cells. But in an inflamed joint the fluid is cloudy and much less viscous.

The rheumatoid synovium synthesizes IgG which may be involved in local immune complex formation. It has been shown that some immune complexes become trapped in the rheumatoid articular cartilage. Samples of this cartilage, when incubated with normal neutrophils, stimulate those neutrophils to become attached and invade the cartilage in an attempt to phagocytose the immune complexes. This can cause damage to cartilage by enzyme-release and generation of oxygen-derived free radicals (Ugai et al, 1983).

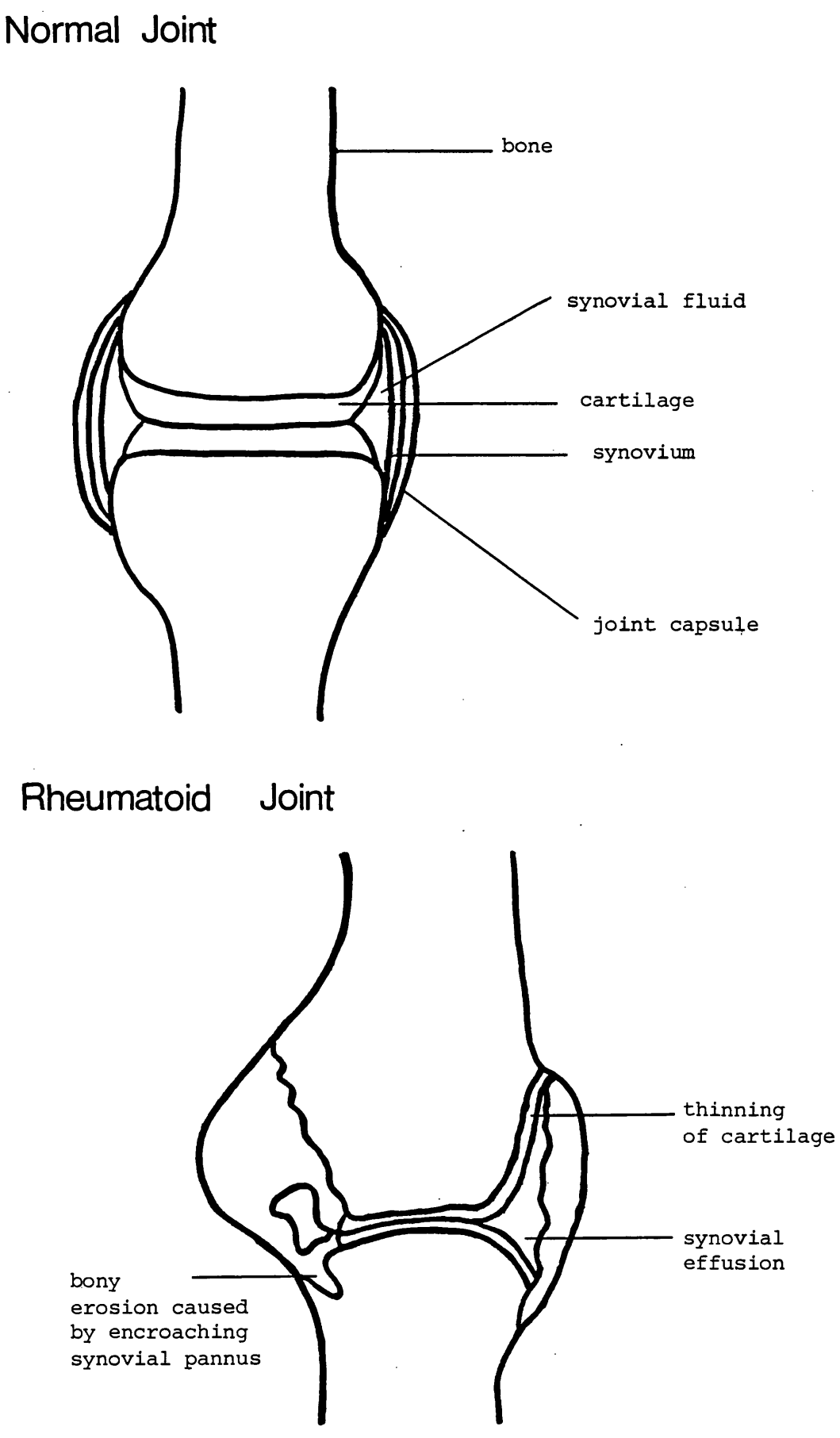


Figure 1.6 Degenerative changes in an inflamed joint

Immune complexes in synovial fluid activate complement and this stimulates an enormous influx of neutrophils. Neutrophils constitute over 90% of cells found in the synovial fluid and leukocyte counts of $50,000/mm^3$ are not unusual. These cells have a half-life of 3 to 4 hours in the joint, thus each day over one billion cells enter the joint to participate in the inflammatory response. Neutrophils ingest the immune complexes with the concomitant release of hydrolytic enzymes (Zvaifler, 1971), oxygen metabolites and leukotriene B_4 (Davidson, Rae and Smith, 1983).

7.1.2 Role of Oxygen-Derived Free Radicals

Oxygen-derived free radicals are capable of mediating a wide variety of tissue damage much of which would be relevant in rheumatoid disease. Oxygen metabolites, either produced enzymatically, from stimulated neutrophils, or by the autooxidation of ferrous salts (Gutteridge, 1982), have been shown to damage cultured endothelial cells (Weiss et al, 1981, Niwa et al, 1982); to cause red cell lysis and liposome oxidation (Kellogg III and Fridovich, 1977); to mediate collagen breakage (Monboisse, Braquet and Borel, 1984); to inhibit serum anti-protease activity (Greenwald, 1981); to depress lymphocyte reactivity, which might explain the disturbed immuno-reactivity of synovial fluid lymphocytes in RA (Niwa et al, 1983); and to damage the phagocytic cells which have produced them (Salin and McCord, 1975; Sweder van Asbeck et al, 1984). Studies in several laboratories have shown that hyaluronic acid is susceptible to oxygen-derived free radical damage (McCord, 1974; Puig-Parallada and Planas, 1978; Del Maestro et al, 1980; Greenwald and Moy, 1980). Hyaluronic acid is the glycosaminoglycan that accounts almost entirely for the viscosity

of synovial fluid and there is good reason to believe that oxygen-derived free radicals (probably $OH^{\bullet}$) bring about depolymerization of hyaluronic acid and thus cause the reduction in synovial fluid viscosity seen in an inflamed joint (Greenwald and Moy, 1980). It is recognised that one mechanism by which neutrophils could damage all these different tissues is by lipid peroxidation induced by radicals (Kellogg III and Fridovich, 1975; Kellogg III and Fridovich, 1977; Dixit, Mukhtar and Bickers, 1982). $OH^{\bullet}$ is capable of initiating lipid peroxidation by its ability to abstract hydrogen atoms from the allylic position of unsaturated lipid to yield hydroperoxides (Figure 1.7) (Badwey and Karnovsky, 1980). The radical ($R^{\bullet}$) formed may then initiate a chain reaction. Singlet oxygen ($^1O_2$) has also been shown capable of mediating lipid peroxidation. As the delta form has an unoccupied outer orbital, it behaves as a strong electrophile and reacts avidly with molecules that contain regions of high electron density such as carbon-carbon double bonds (Badwey and Karnovsky, 1980). The conjugate acid of $O_2^{\bullet -}$, perhydroxyl radical ($HO_2^{\bullet}$) reacts with linolenic, linoleic and arachidonic acids and could also be implicated in oxygen-derived free radical toxicity (Bielski, Arudi and Sutherland, 1983).

It has been suggested (Morehouse et al, 1982) that oxygen-derived free radicals are not the predominant means by which lipid peroxidation occurs, because addition of exogenous catalase to their system <u>in vitro</u> failed to alter the rate of lipid peroxidation. However, this may be artefactual and explained by work by Gutteridge and colleagues who showed that catalase itself can react non-specifically with lipid peroxides, decomposing them as they are formed (Gutteridge, Beand and Quinlan, 1983). Thus, if oxygen-derived free radicals can cause damage in RA via lipid peroxidation, one would expect to see peroxi-

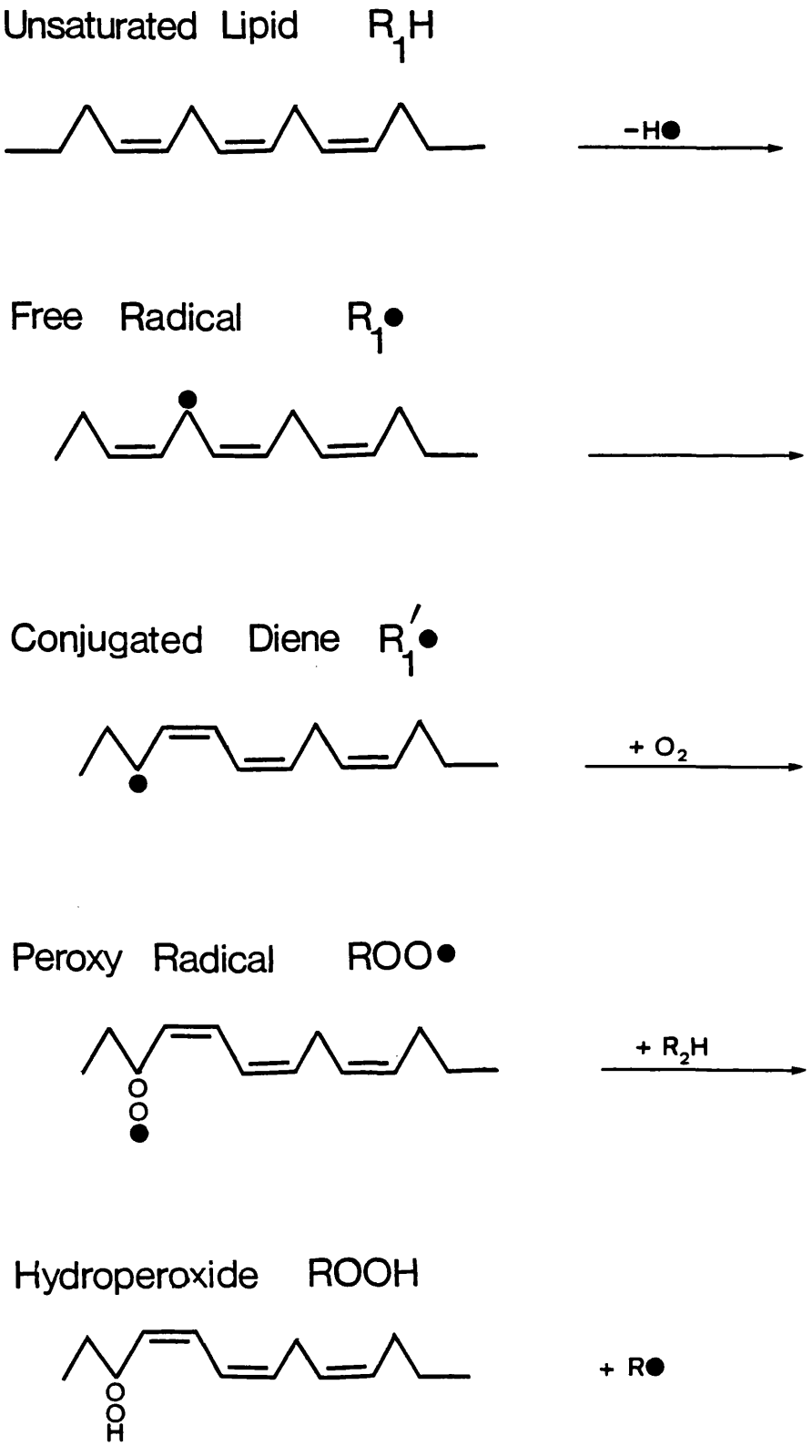


Figure 1.7 OH• mediated lipid peroxidation

dation products in the synovial fluid of RA patients. This is indeed the case, as lipid peroxidation products have been found at increased levels in patients with inflammatory disorders (Lunec et al, 1981; Rowley et al, 1983). In addition, in order for the oxygen-derived free radicals to be implicated in mediating tissue damage, iron would need to be present and has indeed been detected (Gutteridge, Rowley and Halliwell, 1982). It has been found that the amount of lipid peroxidation products present in the synovial fluid of rheumatoid patients correlates with the concentration of iron salts present and with disease activity (Rowley et al, 1983). Moreover, lipid peroxidation is inhibited by the iron chelator desferrioxamine as is iron-catalyzed formation of OH•. High doses of desferrioxamine given intraperitonealy suppresses inflammatory reactions in animal models (Gutteridge, Richmond and Halliwell, 1979; Blake et al, 1983(ii)), further implicating the activity of OH• and iron in mediating tissue damage. Recently it has been reported (Blake et al, 1985(i)) that the administration of intravenous iron dextran to RA patients for the treatment of anaemia resulted in the exacerbation of synovitis, with a concomitant increase in lipid peroxidation products in the synovial fluid.

Oxygen-derived free radicals produced enzymatically or by neutrophils have been shown to be capable of aggregating IgG in vitro (Wickens et al, 1981; Lunec, 1984; Wickens and Dormandy, 1984). Neutrophils may then respond to this aggregated IgG by producing more free radicals and thereby perpetuating the process. The aggregated IgG is identical to fluorescent aggregates isolated from rheumatoid sera and synovial fluids which in turn activates normal neutrophils (Gale et al, 1983). It has also been suggested that IgG aggregates in vivo may be generated by the myeloperoxidase-hydrogen peroxide system (Jasin, 1983).

Neutrophils from patients with rheumatic diseases have been compared with cells from healthy controls with somewhat conflicting results. Niwa and colleagues have found increased oxygen-radical generation from neutrophils from patients with RA as compared with controls (Niwa et al, 1983) and also from patients with Behcets disease which is a form of seronegative arthritis characterized by recurrent oral and genital ulcers (Niwa et al, 1982). However, others have found no difference in the $O_2^{\bullet -}$ generating power of neutrophils from RA patients and controls (Chui et al, 1983; Minty et al, 1983), but have found a depression in $O_2^{\bullet -}$ production by neutrophils from patients with Felty's syndrome which is a complication of severe RA characterized by neutropenia and splenomegaly (Chui et al, 1983). Neutrophils from patients with rheumatoid arthritis show a marked reduction in directed motility. This is thought to be due to immunoglobulin inclusions as the number of inclusions in cells from these patients is inversely proportional to their chemotactic activity (Goddard et al, 1984).

7.2 Progressive Systemic Sclerosis

P.S.S. is a connective tissue disease of unknown aetiology that is characterized by the excessive accumulation of collagen in the skin and internal organs such as oesophagus, heart, lungs and kidneys (Jimenez, 1983). This accumulation of collagen is thought to involve an alteration in the regulation of collagen biosynthesis by fibroblasts. The fibroblasts may be directly activated or else some factor in the disease is causing a process of selection of fibroblasts programmed to producing increased amounts of collagen (Botstein, Sherer and LeRoy, 1982). The skin lesion evolves through an oedematous phase containing

lymphocyte infiltrates and culminates in a taut, hidebound appearance characteristic of fibrosis.

There may be a poly-arthritis affecting the small joints of the hands and soft tissue swelling due to a subacute synovitis. Patients with P.S.S. frequently have Raynaud's phenomenon (Buckingham et al, 1980). The disease usually presents between 30-50 years of age and women are affected about twice as often as men. About 30% of patients have skin involvement localized to distal extremities (sclerodactyly); 30% have skin involvement localized to mid-forearm, mid-calf or face (acrosclerosis); 15-20% of patients, as well as having distal involvement have involvement of the trunk (diffuse disease). This latter group have the more severe systemic disease (Maddison,Personal Communication). Serological abnormalities include the presence of antinuclear and other auto-antibodies (Whiteside et al, 1983). Pathological abnormalities include damage to the endothelial cells of the microvasculature, arterial changes and abnormalities in capillary structure (Shanahan Jr., and Korn, 1982). It has been suggested that there is a functional defect in serum protease inhibitors (Kahaleh and LeRoy, 1983) and a build up of a serum protease cytotoxic factor active against endothelial cells is reported in 50% of cases (Shanahan Jr. and Korn, 1982; Cohen, Johnson and Hurd, 1983). It has also been reported that the factor responsible for endothelial cell cytotoxicity may be an oxidized lipoprotein (Blake et al, 1985(ii)). Also reported is the occurrence of increased frequency of chromosome breakage and rearrangement in cell cultures of blood and skin from P.S.S. patients (Sherer et al, 1981).

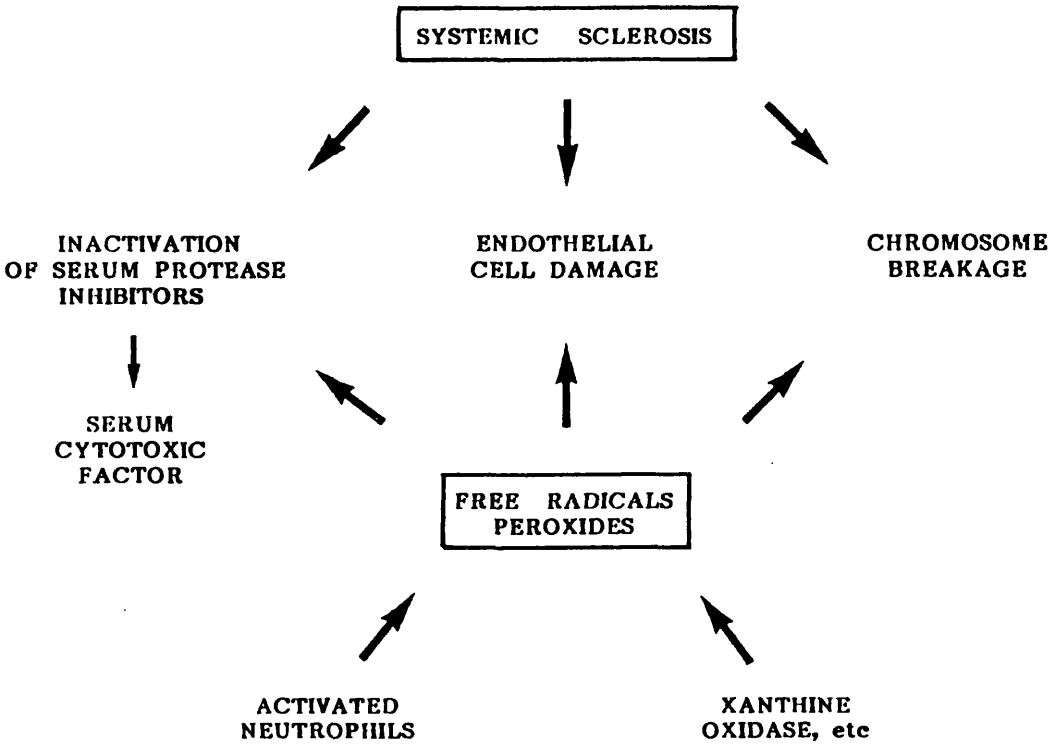


Figure 1.8 Implication of free radicals and peroxides in systemic sclerosis.

7.2.1 Implication of O_2-Derived Free Radicals and Products of Arachidonic Acid Metabolism in the Pathogenesis of P.S.S.

How O_2-derived free radicals may be implicated in P.S.S. is shown in Figure 1.8. Free radicals, either generated enzymatically or derived from activated neutrophils are capable of mediating endothelial cell damage (Del Maestro et al, 1980; Weiss et al, 1981). One of the mechanisms for functional defects in serum protease inhibitors is by direct damage by neutrophil-derived oxygen free radicals, which would lead to a build up of serum cytotoxic factor (Carp and Janoff, 1979). If oxidized lipoprotein is responsible for cytotoxicity, neutrophil-derived free radicals are suitable oxidants. It has also been shown that DNA suffers covalent bond cleavage on exposure to hydroxyl radicals (Mello Filho and Meneghini, 1984). It has been reported that patients receiving the anti-neoplastic drug bleomycin have developed a cutaneous fibrosis"indistinguishable from that encountered in P.S.S." (Finch et al, 1979), although this point appears contentious. Bleomycin is known to generate free radicals and also induces chromosome cleavage (Tom and Montgomery, 1980) which can be inhibited by SOD (Galvan et al, 1981).

8. INHIBITORS OF OXYGEN-DERIVED FREE RADICAL-MEDIATED DAMAGE IN VIVO

8.1 SOD

Superoxide dismutase was first described by McCord and Fridovich in 1969 who recognised it as the active enzyme in copper-containing proteins isolated from human and bovine sources. They demonstrated its use in preventing $O_2^{\cdot-}$-mediated reactions. As the product of the dismutation of $O_2^{\cdot-}$ which it catalyses is H_2O_2, also a potentially

hazardous agent, its only value in vivo would seem to be in the prevention of the formation of $OH^{\bullet}$, formed in the reaction between $O_2^{\bullet -}$ and H_2O_2. SOD has been given intravenously and shown to be effective against various inflammatory diseases such as RA, dermatomyositis and lupus erythematosus (Bragt, 1984). Intra-synovial administration of SOD in the form of orgotein, the generic name for drug versions of CuZn superoxide dismutases has proved effective in relieving symptoms of RA (Goebel, Storck and Neurath, 1981). However, in vivo, the protection offered by extracellular endogenous SOD is minimal. SOD is either completely absent (Blake et al, 1981) from rheumatoid synovial fluid, or else is present in negligible amounts (Biemond, Swaak and Koster, 1984). In recent experiments, i.v. administration of rat SOD to rats with adjuvant arthritis has proved ineffective as an anti-inflammatory agent. Rat SOD is manganese-containing, and it is now suggested that the anti-inflammatory activity of CuZn SOD is due to the presence of copper (Halliwell, Personal Communication).

8.2 Catalase

Catalase is capable of detoxifying H_2O_2, reducing it to water:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

However, Blake et al found low concentrations of catalase in RA synovial fluid and, although Biemond et al found a significant increase in the levels of catalase in RA synovial fluids compared with controls, the concentration is too low to expect considerable protection against H_2O_2 in synovial fluid, (Blake et al, 1981; Biemond, Swaak and Koster, 1984).

8.3 Caeruloplasmin

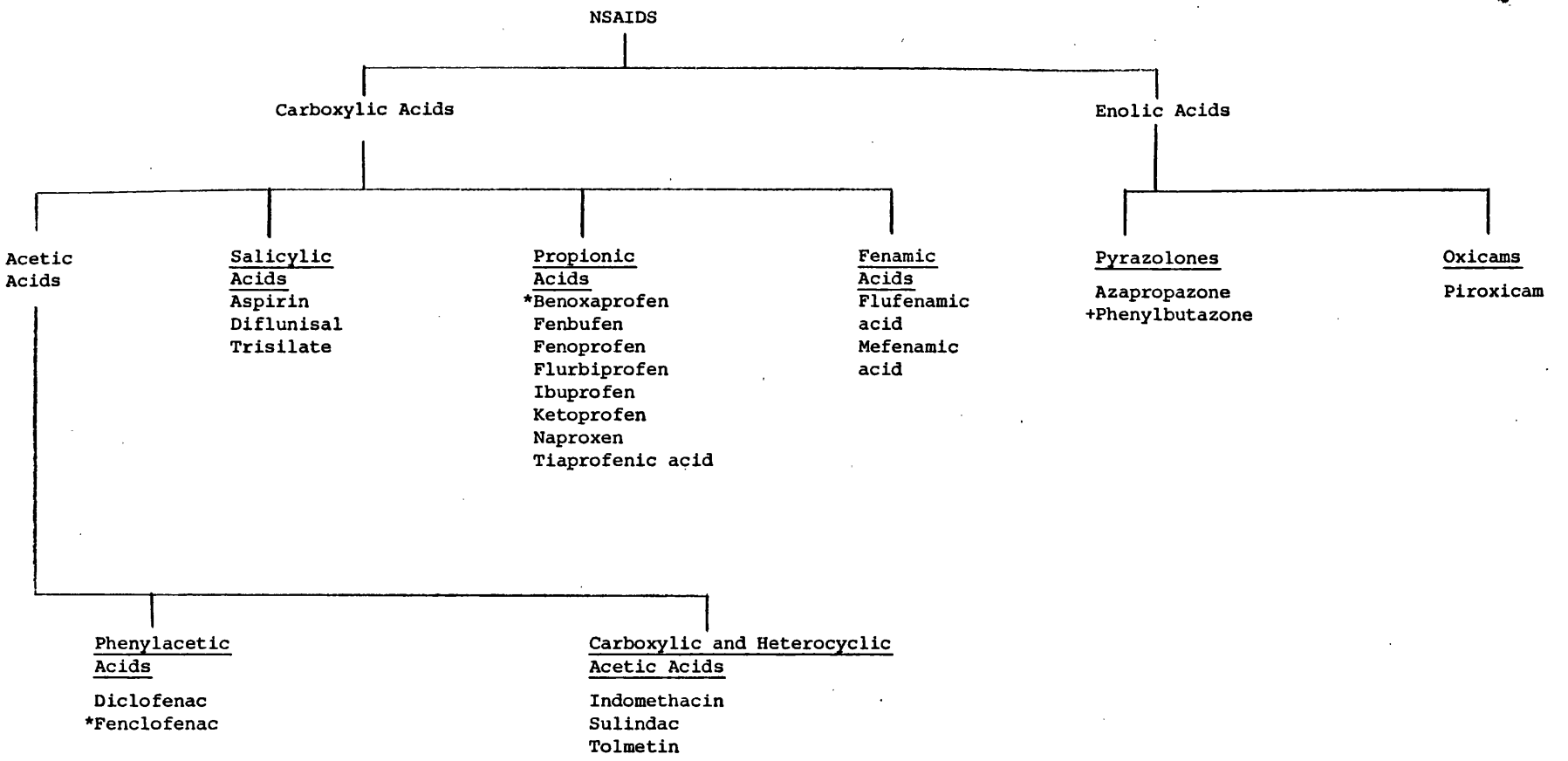
It was originally thought that caeruloplasmin, the copper-containing protein, an acute-phase reactant, had a SOD-like activity in that it scavenged $O_2^{\bullet -}$ radicals (Goldstein et al, 1979; Goldstein et al, 1982). However, this claim has been refuted by many groups. Bannister et al found that the rate of disappearance of $O_2^{\bullet -}$ is not markedly increased by the presence of caeruloplasmin and that there was no evidence for any accelerated dismutation comparable to that caused by SOD (Bannister et al, 1980). It is now agreed that the anti-oxidant activity of caeruloplasmin is due to its ferroxidase activity. It inhibits ferrous ion-stimulated lipid peroxidation and ferrous ion-dependent formation of $OH^{\bullet}$ by catalysing the reaction $Fe^{2+} \longrightarrow Fe^{3+}$. Thus any free iron in extracellular fluids would be kept in Fe^{3+} state (Gutteridge, Richmond and Halliwell, 1980; Gutteridge, 1983). It also prevents copper ions from stimulating lipid peroxidation. It is present in elevated amounts in sera from RA patients compared with controls (Blake et al, 1983(i); Biemond, Swaak and Koster, 1984) and so it may act as a protective factor, but although synovial fluid from RA patients contains caeruloplasmin, its ferroxidase activity is low. This deficiency may contribute to radical damage in the joint.

8.4 Vitamin E

Vitamin E is a powerful anti-oxidant *in vitro*, but its importance in this capacity *in vivo* in man is questionable. In animals, vitamin E deficiency is always associated with an increased susceptability to free-radical oxidation and at least one human disease - a haemolytic-thrombocytopenic syndrome in prematurely born infants - responds to vitamin E (Dormandy, 1978).

9. ACTION OF NSAIDS AND INHIBITORS OF ARACHIDONIC ACID METABOLISM ON NEUTROPHIL FUNCTION

There are many non-steroidal anti-inflammatory drugs used in the treatment of RA to reduce inflammation and pain (see Figure 1.9). The possible action of these drugs *in vitro* on phagocytic cell function as well as on synovial fluid degradation induced by free radicals has been the subject of many studies. It is widely believed that the ability of these drugs to suppress inflammation is due to their action as cyclooxygenase inhibitors, for PGE_2 plays a major role in the development of inflammatory erythema, oedema and pain (Higgs and Vane, 1983). However, this alone would not be effective in preventing the inflammation caused by other mediators which are released from activated phagocytic cells and NSAIDS may act in other ways on the cells. The results of the studies are conflicting and often depend upon the type and origin of the cells used, the stimulus employed and the concentration range of the drug in question. These are all summarized in Table 1.1. Generally it has been found that, *in vitro*, benoxaprofen (lipoxygenase/cyclooxygenase inhibitor) inhibits neutrophil chemotaxis and phagocytosis (Wildfeuer, 1983; Anderson et al, 1984; Turner et al, 1984). Diclofenac sodium and phenylbutazone (cyclooxygenase inhibitors) also inhibit chemotaxis (Perianin, Labro and Hakim, 1982; Wildfeuer, 1983) whilst aspirin and indomethacin (cyclooxygenase inhibitors) have no effect on chemotaxis (Wildfeuer, 1983). Piroxicam (cyclooxygenase inhibitor) inhibits neutrophil aggregation (Edelson et al, 1982). Moreover piroxicam, indomethacin, flurbiprofen, ibuprofen, ketoprofen (cyclooxygenase inhibitors), benoxaprofen, eicosatetraynoic acid, (ETYA) (mixed cyclooxygenase/lipoxygenase inhibitors) and p-bromophenacyl bromide (pBPB) (phospholipase inhibitor) all inhibit degranulation (Smith and Iden, 1980; Smolen and Weissmann, 1980; Edelson et al, 1982).



*Withdrawn by the committee on safety of medicines.

+Available only from hospitals for treatment of ankylosing spondylitis.

FIGURE 1.9 Classification of some of the more commonly used NSAIDS

DRUG	CONCn RANGE	CELL TYPE	STIMULUS	EFFECT	COMMENTS	REFERENCE
Benoxaprofen	5×10^{-6}M - 10^{-3}M	Human PMN	FMLP	Inhibits chemotaxis	Statistically significant at $>10^{-4}$M	Anderson et al 1984
Benoxaprofen	5×10^{-6}M - 10^{-3}M	Human PMN	C.albicans	No effect on phagocytosis		Anderson et al 1984
Benoxaprofen	10^{-6}M - 10^{-3}M	Human PMN	HAGG	Inhibits phagocytosis	Statistically significant at $>10^{-4}$M	Turner et al 1983
Benoxaprofen	10^{-6}M - 10^{-3}M	Human PMN	FMLP	Inhibits chemotaxis	Only at 10^{-3}M	Turner et al 1983
Benoxaprofen	10^{-4}M - 10^{-2}M	Human PMN	Autologous Serum + C.alb.	Inhibits migration No effect on $O_2^{\bar{\bullet}}$ release		Wildfeuer 1983
Benoxaprofen	10^{-3}M - 10^{-2}M	Human MØ	Autologous Serum + C.alb.	Inhibits migration No effect on $O_2^{\bar{\bullet}}$ release		Wildfeuer 1983
Benoxaprofen	5×10^{-5}M - 2.5×10^{-4}M	Human PMN	FMLP	Inhibits degranulation	$p<0.01$	Smith & Iden 1980
Diclofenac Sodium	6×10^{-4}M	Human PMN	Autologous Serum + C.alb.	Inhibits migration No effect on $O_2^{\bar{\bullet}}$ release		Wildfeuer 1983
Diclofenac Sodium	6×10^{-4}M	Human MØ	Autologous Serum + C.alb.	Inhibits migration No effect on $O_2^{\bar{\bullet}}$ release		Wildfeuer 1983
Diclofenac Sodium	4×10^{-8}M - 4×10^{-4}M	Guinea Pig Macrophage	None	Inhibits $O_2^{\bar{\bullet}}$ release	ID_{50} 4×10^{-7}M	Oyanagui 1976
Diclofenac Sodium	4×10^{-6}M - 2.5×10^{-3}M	Guinea Pig Macrophage	None	Inhibits $O_2^{\bar{\bullet}}$ release	ID_{50} 2×10^{-5}M	Oyanagui 1978

DRUG	CONCn RANGE	CELL TYPE	STIMULUS	EFFECT	COMMENTS	REFERENCE
Phenylbutazone	1.7×10^{-5}M - 1.7×10^{-4}M	Human PMN	FMLP	Inhibits migration		Perianin, Labro & Hakim, 1982
Phenylbutazone	4×10^{-6}M- 4×10^{-2}M	Guinea Pig Macrophage	None	Inhibits $O_2^{\bullet-}$	ID_{50} 4×10^{-5}M	Oyanagui 1976
Indomethacin	6×10^{-4}M	Human PMN	Autologous Serum + C.alb.	No effect on migration No effect on $O_2^{\bullet-}$ release		Wildfeuer 1983
Indomethacin	5×10^{-5}M	Human PMN	FMLP OZ	Inhibits degranulation by 50% No effect on degranulation		Smolen & Weissman 1980
Indomethacin	10^{-6}M - 10^{-4}M	Human PMN	OZ, PMA	No effect on $O_2^{\bullet-}$ release		Spisani et al 1984
Indomethacin	10^{-7}M - 10^{-4}M	Guinea Pig Macrophage	None	Inhibits $O_2^{\bullet-}$ release	ID_{50} 10^{-5}M	Oyanagui 1976
Indomethacin	4×10^{-6}M - 2.5×10^{-4}M	Guinea Pig peritoneal exudate cells	None	Inhibits $O_2^{\bullet-}$ release	ID_{50} 3×10^{-5}M	Oyanagui 1978
Indomethacin	5×10^{-5}M - 2.5×10^{-4}M	Human PMN	FMLP	Inhibits degranulation	$p<0.01$	Smith & Iden 1980
Piroxicam	2.5×10^{-5}M - 10^{-4}M	Human PMN	FMLP	Inhibits aggregation	ID_{50} 5×10^{-5}M	Edelson et al 1982
Piroxicam	5×10^{-5}M	Human PMN	FMLP	Inhibits degranulation		Edelson et al 1982
Piroxicam	5×10^{-5}M	Human PMN	ConA, PMA	No effect on degranulation		Edelson et al 1982
Piroxicam	5×10^{-5}M	Human PMN	FMLP, ConA, PMA	Inhibits $O_2^{\bullet-}$ release		Edelson et al 1982
Piroxicam	2.5×10^{-5}M - 10^{-4}M	Human PMN	FMLP	Inhibits aggregation	ID_{50} 5×10^{-5}M	Abramson et al 1983

DRUG	CONCn RANGE	CELL TYPE	STIMULUS	EFFECT	COMMENTS	REFERENCE
Piroxicam	5×10^{-5}M	Human PMN	FMLP	Inhibits $O_2^{\bullet-}$ release & degranulation	$p<0.01$	Abramson et al 1983
Piroxicam	5×10^{-5}M	Human PMN	ConA) PMA)	Inhibits $O_2^{\bullet-}$ release but not degranulation	$p<0.01$	Abramson et al 1983
Mefenamic Acid	10^{-6} - 10^{-4}M	Guinea Pig Macrophage	None	Inhibits $O_2^{\bullet-}$	ID_{50} 7×10^{-5}M	Oyanagui 1976
Mefenamic Acid	1.6×10^{-5}M - 10^{-3}M	Guinea Pig peritoneal exudate cells	None	Inhibits $O_2^{\bullet-}$	ID_{50} 10^{-4}M	Oyanagui 1978
Flufenamic Acid	8×10^{-5}M - 8×10^{-3}M	Guinea Pig Macrophage	None	Inhibits $O_2^{\bullet-}$	ID_{50} 8×10^{-5}M	Oyanagui 1976
Ibuprofen	1.6×10^{-5}M - 10^{-3}M	Guinea Pig peritoneal exudate cells	None	Inhibits $O_2^{\bullet-}$	ID_{50} 10^{-4}M	Oyanagui 1978
Ibuprofen	4×10^{-6}M - 4×10^{-4}M	Human PMN	FMLP	Inhibits aggregation	ID_{50} 4×10^{-5}M	Abramson et al 1983
Ibuprofen	4×10^{-5}M	Human PMN	FMLP	Inhibits degranulation, not $O_2^{\bullet-}$ release	$p<0.01$	Abramson 1983
Ibuprofen	4×10^{-5}M	Human PMN	ConA) PMA)	No effect on degranulation or $O_2^{\bullet-}$ release		Abramson et al 1983
Ibuprofen	5×10^{-5}M - 2.5×10^{-4}M	Human PMN	FMLP	Inhibits degranulation	$p<0.05$	Smith & Iden 1980
Flurbiprofen	5×10^{-5}M - 2.5×10^{-4}M	Human PMN	FMLP	Inhibits degranulation	$p<0.05$	Smith & Iden 1980
Ketoprofen	5×10^{-5}M - 2.5×10^{-4}M	Human PMN	FMLP	Inhibits degranulation	$p<0.05$	Smith & Iden 1980

DRUG	CONCn RANGE	CELL TYPE	STIMULUS	EFFECT	COMMENTS	REFERENCE
Aspirin	200mcg/ml	Human PMN	Autologous Serum + C.alb.	No effect on migration or $O_2^{\bar{\cdot}}$ release		Wildfeuer 1983
Aspirin	$3x10^{-6}$ - $3x10^{-2}$M	Guinea Pig Macrophage	None	Inhibits $O_2^{\bar{\cdot}}$ release	ID_{50} $3x10^{-4}$M	Oyanagui 1976
Aspirin	$2.5x10^{-4}$M - 10^{-3}M	Guinea Pig peritoneal exudate cells	None	Inhibits $O_2^{\bar{\cdot}}$ release	ID_{50} > 10^{-3}M	Oyanagui 1978
Aspirin	$5x10^{-5}$M - $2.5x10^{-4}$M	Human PMN	FMLP	No effect on degranulation		Smith & Iden 1980
ETYA	$1.2x10^{-5}$M	Human PMN	FMLP	Inhibits degranulation by 50%		Smolen & Weissmann 1980
ETYA	$1.2x10^{-5}$M	Human PMN	OZ	No effect on degranulation		Smolen & Weissmann 1980
pBPB	$8x10^{-6}$M	Human PMN	FMLP	Inhibits degranulation by 50%		Smolen & Weissmann 1980
pBPB	$8x10^{-6}$M	Human PMN	FMLP	Inhibits $O_2^{\bar{\cdot}}$ release		Smolen & Weissmann 1980

TABLE 1.1 The effects of NSAIDS and other inhibitors on phagocytic cell function.

The evidence for whether or not $O_2^{\bullet-}$ generation is affected by these agents is more contentious, with some workers claiming that NSAIDS such as benoxaprofen, diclofenac, aspirin and indomethacin have no effect (Wildfeuer, 1983; Spisani et al, 1984), whilst other laboratories claim that these same drugs along with other cyclooxygenase inhibitors such as phenylbutazone, mefenamic acid, flufenamic acid, piroxicam, ibuprofen and phospholipase inhibitor pBPB all inhibit $O_2^{\bullet-}$ release (Oyanagui, 1976, 1978; Smolen and Weissmann, 1980; Edelson et al, 1982). The discrepancies in these results could be explained in terms of the wide range of concentrations of drugs used and the stimulus employed as well as the type and origin of the cells. Certainly most of the concentrations used are too high to be relevant in vivo, especially as it must be remembered that effective plasma concentrations of NSAIDS are affected by plasma proteins and that normally at least 90% of the drug will be protein-bound, although protein-binding is reported to be lower in synovial fluid (Wanwimolruk, Brooks and Birkett, 1983). The concentration in the plasma will normally be about 10^{-7} - 10^{-6}M, with benoxaprofen rather higher at 10^{-4}M (Anderson et al, 1984). Thus, from Table 1.1, it can be seen that none of the experiments showing inhibition of human phagocyte cell function are performed at physiologically relevant concentrations, except for Smith and Iden's work with benoxaprofen showing significant inhibition of degranulation at 5×10^{-5}M (Smith and Iden, 1980). Only Oyanagui (1976, 1978) has been able to show consistently that $O_2^{\bullet-}$ release is inhibited by all NSAIDS he tested, but this is using guinea pig macrophages and a rather obscure assay method which employs NADH and lactate dehydrogenase. He did not use standard assays because they proved too insensitive and thus the amounts of $O_2^{\bullet-}$ he was measuring must have been minute.

There are few published reports on the functions of neutrophils isolated from individuals following administration of NSAIDS. Anderson et al (1984) reports that coincubation of normal neutrophils with serum from individuals who had ingested benoxaprofen, 600mg daily for 7 days, resulted in decreased cell migration (mean inhibition 44%). However, neutrophils from those individuals showed no effects of decreased migration. This was attributed to the washing of the drug off the neutrophils during cell processing. Ingestion of benoxaprofen was not associated with any detectable effects on phagocytosis or oxidative metabolism by neutrophils. Similarly Wildfeuer (1983) found that benoxaprofen did not significantly reduce the chemotactic locomotion of neutrophils or monocytes isolated from volunteers after repeated oral administration of the drug (600mg daily for 7 days). Abramson et al (1983) reported that, following ingestion of piroxicam daily for 3 days, neutrophils from those individuals, stimulated with FMLP, showed decreased aggregation, $O_2^{\bullet -}$ release and degranulation. The responses of FMLP-stimulated neutrophils from subjects taking ibuprofen at 2400mg daily for 3 days showed inhibition of aggregation and degranulation but there was no effect on superoxide generation.

The mechanism of action of these drugs in inhibiting neutrophil functions is not clear. Various suggestions have been put forward including inhibition of membrane calcium release (Edelson et al, 1982); blockage of membrane SH groups or NADPH oxidase (Oyanagui, 1978) and elevation of cyclic AMP (Higgs and Vane, 1983). However the doses which tend to produce these effects are too high to be relevant in vivo. Moreover, it may be that some of these NSAIDS appear to be inhibiting neutrophil function merely by interfering with the binding of the stimulus to its receptor. Many of these studies used FMLP to stimulate the neutrophils and yet it has been shown that piroxicam (Edelson et

al, 1982); indomethacin (Abita, 1981) and sulindac (Van Dyke et al, 1982) are all capable of inhibiting binding of [^{3}H] FMLP. Another suggestion is that these drugs may act as direct O_2-derived free radical scavengers. It has been proposed that NSAIDS may act via the formation of complexes with copper in vivo which can catalytically eliminate superoxide radicals (Lengfelder, 1984) and indeed copper complexes of indomethacin following in vivo administration of the drug have been isolated. NSAIDS have been shown to inhibit synovial fluid degradation induced by free radicals generated enzymatically (Puig, Parellada and Planas, 1978) and it has also been shown that NSAIDS reduce the levels of free radical oxidation products in the serum and synovial fluid of patients with RA (Lunec et al, 1981). Moreover the inactivation of a bacteriophage by OH• radicals is strongly inhibited by the presence of NSAIDS. Pulse radiolysis shows that OH• reacts rapidly with the drugs (Hiller and Wilson, 1983).

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

All chemicals were supplied by British Drug Houses Ltd., Poole unless otherwise stated.

Material	Supplier
Benoxaprofen BW 755C	Lilly Research Centre Ltd
Diclofenac + analogues Pirprofen	Ciba Geigy Ltd Horsham, W. Sussex
Fenclofenac + analogues	Reckitt and Coleman Hull
Piroxicam	Pfizer Ltd Sandwich, Kent
Antisera p-Bromophenacyl bromide (pBPB) Catalase (bovine liver)	Sigma Fine Chemicals Poole
Cytochrome C Type III (horse heart)	
Dimethyl sulphoxide (DMSO)	
5,5' Dithiobis (2-nitrobenzoic acid) (DTNB)	
n-Formyl-l-methionyl-l-leucyl-l- phenylalanine (FMLP)	
Horseradish peroxidase (HRPO) Type II	
p-Hydroxymercuriphenylsulphonic acid (pHMPSA)	
Indomethacin	
Nordihydroguaiaretic acid (NDGA)	
Quinacrine	
Superoxide dismutase (SOD) (bovine blood)	
Xanthine	
Xanthine oxidase Grade 1 (buttermilk)	
Zymosan A	

MATERIALS Continued

Material	Supplier
Calf blood erythrocytes in Alsevers preservative	Tissue Culture Services Slough, Berks
Heparin Sodium 1000U/ml	Weddel Pharmaceuticals Ltd Wrexham
Phosphate buffered saline (Dulbecco 'A') tablets	Oxoid Basingstoke, Hants
DEAE - Sephadex A-50 Protein A - Sepharose CL-4B Percoll	Pharmacia GB Ltd Milton Keynes
Nunc polycarbonate round-bottomed centrifuge tubes 10ml Nunc 96 microwell flat-bottomed plates	Gibco Europe Ltd Paisley
LP3, LP4 tubes	Luckham Ltd Burgess Hill, Surrey
Brunswick blood collection tubes	Sherwood Medical Industries Ltd Crawley, Surrey.

Equipment	Supplier
Apple IIe computer	Apple Computer Inc. Cupertino, California
CE292 Digital UV Spectrophotometer	Cecil Instruments Ltd Cambridge
MR580 Microelisa Auto Reader	Dynatech Labs Inc. Alexandria, Virginia
Oxygen Electrode	Hansatech Ltd Kings Lynn, Norfolk
Heraeus Christ Labofuge 6000 Centrifuge	V. Howe Ltd Wandsworth, London
CR650S Chart recorder	J. J. Instruments Southampton
SM-Lux Westlar Microscope	E. Leitz (Instruments) Ltd Luton
Electrophoresis tank	Shandon Southern Ltd Runcorn, Cheshire

ROUTINE BUFFERS AND SOLUTIONS

Calcium and Magnesium Free Salt Solution (CMFSS)

Sodium Chloride		8.000g
Potassium Chloride		0.400g
Potassium Dihydrogen Phosphate		0.060g
Disodium Hydrogen Phosphate		0.048g
Distilled Water	to	1000 ml

The pH was adjusted to 7.3 with 1M NaOH, then the buffer was aliquoted in 100ml quantities, autoclaved at 120°C for 20 minutes, and stored at 4°C. On the day of use, 0.160g of glucose was added to each 100ml quantity.

Buffered Phenol Red Solution

Sodium Chloride		1.0228g
Potassium Dihydrogen Phosphate		0.1700g
Glucose		0.1238g
Phenol Red		0.0100g
Horseradish Peroxidase		850 Units
Distilled Water	to	100ml

The pH was adjusted to 7.3 with 1M NaOH. This solution was prepared immediately prior to use and not stored.

Phosphate Buffer 0.01M

Potassium Dihydrogen Phosphate		1.74g
Disodium Hydrogen Phosphate		1.78g
Distilled Water	to	1000 ml

The pH was adjusted to 6.5 with 1M NaOH. The buffer was prepared immediately prior to use and not stored.

Phosphate Buffered Saline (PBS)

This buffer was prepared from commercially available tablets, either as single strength (1 tablet in 100ml distilled water) or 10X strength (10 tablets in 100ml distilled water). When used in the preparation of Percoll it was first filter-sterilized using a millipore filter pore size 0.22µm.

Ammonium Chloride Solution 0.83% w/v

Ammonium Chloride		0.083g
Distilled Water	to	10 ml

This was used for the lysis of contaminating erythrocytes during neutrophil preparation. It was prepared immediately prior to use and not stored.

SOURCE OF EXPERIMENTAL BLOOD AND PATIENT SELECTION

The majority of experiments and all control experiments were performed with cells obtained from the blood of normal healthy volunteers. These volunteers were either staff and students of Bath Arthritis Research Centre or staff of the Royal National Hospital for Rheumatic Diseases (RNHRD), Bath.

Patients with progressive systemic sclerosis (PSS) were selected by Dr. Peter Maddison, Consultant Rheumatologist. Blood was obtained from age- and sex-matched patients with rheumatoid arthritis receiving a similar drug therapy to those with PSS. These were all in-patients from the wards of RNHRD.

Patients with peripheral vascular disease were identified by Dr. Tony Woolf, Senior Registrar, RNHRD. These were all patients of Mr. R. Turnball, Consultant Surgeon, and were in-patients at the Royal United Hospital, Bath.

Patients used in the non-steroidal anti-inflammatory drugs *in vivo* study were either early synovitis patients under the care of Dr. Tony Woolf, or else osteoarthritis patients under the care of Dr. Jane Calin.

All blood was obtained by venepuncture with informed consent.

METHODS

1. PREPARATION OF CELLS

Cell separation was performed in round-bottomed polycarbonate centrifuge tubes to prevent activation of the neutrophils. These tubes, unlike those made of glass, do not promote adherence of neutrophils (Cook, Counter and McColley, 1976).

The colloidal silica sol coated with polyvinylpyrrolidone marketed as Percoll was used to separate cells by density-gradient centrifugation (Pertoft, Back and Lindahl-Kiessling, 1968; Pertoft et al, 1977; Segal, Fortunato and Herd, 1980).

1a) Preparation of Percoll

Percoll from Pharmacia has a density of 1.130 ±0.005g.ml^{-1}. Nine parts of this were mixed with one part of 10X PBS to give "100% Percoll". This was then further diluted with single strength PBS to give the appropriate densities for separating the required cell populations from heparinized whole human peripheral blood. These were:

77% v/v (density 1.097g.ml^{-1}) granulocytes

65% v/v (density 1.083g.ml^{-1}) mononuclear cells

52% v/v (density 1.068g.ml^{-1}) monocytes

1b) Cell Separation

Cells were prepared from whole human peripheral blood, with heparin as the anti-coagulant at a concentration of 125U per 10ml of blood. The blood was diluted X2 with CMFSS.

Gradients of Percoll were prepared by placing 2ml 77%v/v Percoll in the centrifuge tube and layering onto this, 1.6ml of 65%v/v Percoll. This was accomplished with the aid of a 2ml syringe barrel fitted with a 21G needle, the syringe being clamped so that the tip of the needle just rested on the meniscus of the 77%v/v Percoll.

The 65%v/v Percoll was then placed in the syringe with a pasteur pipette, and allowed to run onto the top of the 77%v/v Percoll. The diluted blood was then layered on top of the Percoll gradient using a bent pasteur pipette. The tubes were centrifuged at room temperature at 400g for 30 minutes.

This procedure resulted in 2 discrete bands of cells (Figure 2.1). Granulocytes were harvested from the 77%v/v/65%v/v Percoll interface and the mononuclear cells were harvested from the 65%v/v Percoll/plasma interface using pasteur pipettes. Granulocytes were washed in CMFSS by centrifugation at 250g for 10 minutes. Any contaminating erythrocytes were then lysed by treatment with 0.83% w/v ammonium chloride at 37°C for 10 minutes.

If monocytes were to be prepared, the mononuclear cells were suspended in CMFSS and layered on top of 2ml 52% v/v Percoll. The tubes were then centrifuged at room temperature at 750g for 30 minutes and the band of monocytes harvested from the surface of the 52% v/v Percoll. Both cell populations were then washed twice in CMFSS by resuspension and pelleting at 250g for 10 minutes and the resultant pellets resuspended in 1ml of appropriate buffer for counting.

All cell counts were made using a Neubauer counting chamber. Cell suspensions were diluted in white cell counting fluid containing 3% acetic acid and 0.1% methylene blue. Cell viabilities were checked by the method of trypan blue exclusion.

Cells prepared in this way were always >95% pure and >99% viable. The granulocytes were considered to contain a negligible number of eosinophils and basophils and will therefore be termed "neutrophils" from now on in the text.

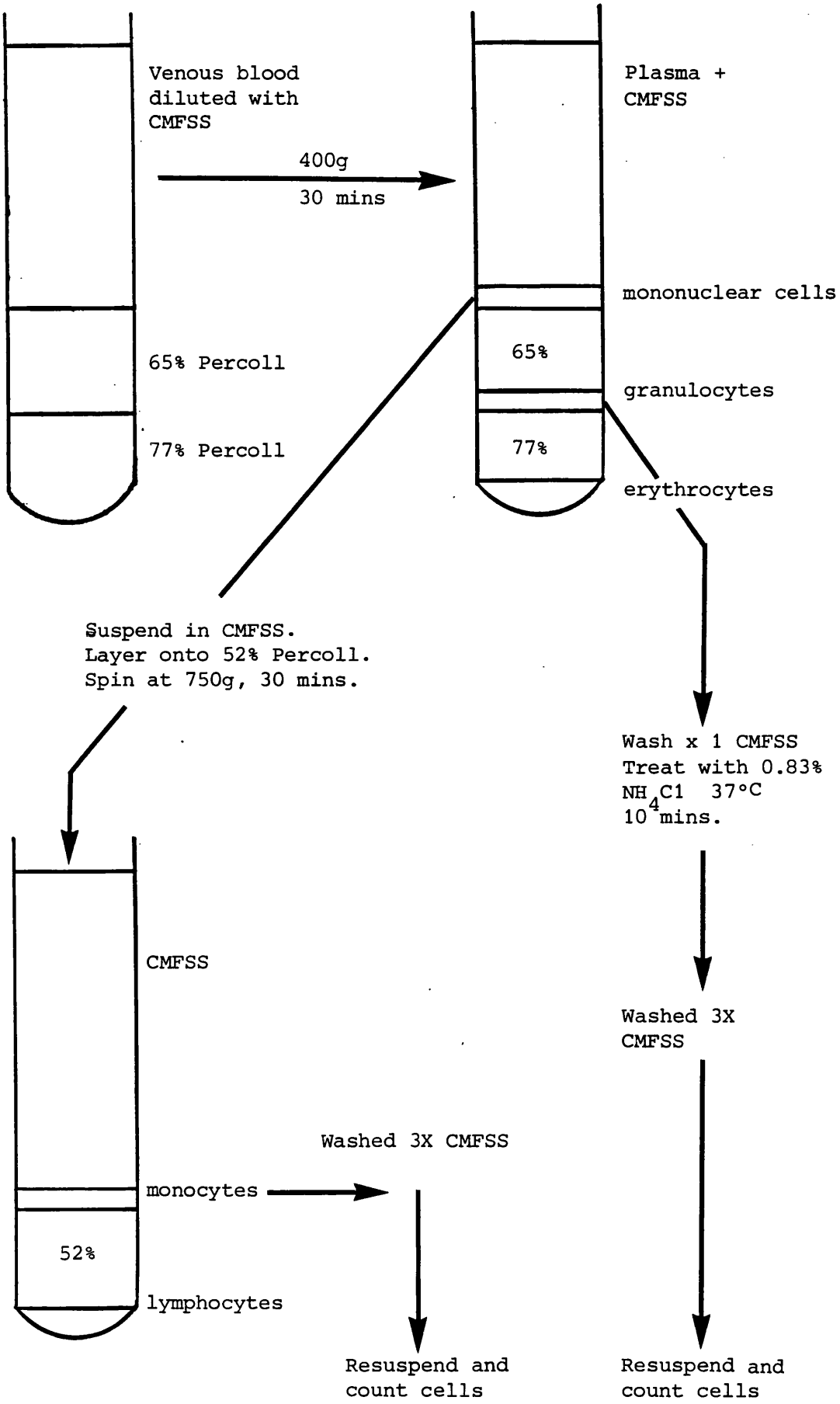


Figure 2.1 CELL SEPARATION

2. PREPARATION OF STIMULI

During the course of these studies, three stimuli were employed. These were heat-aggregated IgG (HAGG); n-formyl-l-methionyl-l-leucyl-l-phenyl-alanine (FMLP) and opsonized zymosan.

2a) Heat-aggregated IgG

Preparation of IgG

Ion-exchange chromatography with diethyl aminoethyl (DEAE) Sephadex was employed to prepare IgG in a modification of a method by Ishizaka (1965). 10ml of an IgG myeloma serum were dialysed overnight against 10mM phosphate buffer pH 6.5. DEAE-Sephadex was swollen in 10mM phosphate buffer pH 6.5 overnight. After filtration through a Buchner funnel, the cake of Sephadex was mixed with the serum to give a thick paste which was stirred for 1 hour at 4°C. It was then filtered again and washed with phosphate buffer. The filtrate was collected and the protein content determined by the Folin-Ciocalteau method of protein estimation (Lowry 1951). The IgG solution was aliquoted into 1ml quantities and stored at -20°C.

Immuno-electrophoresis

The components of the two batches of IgG solution prepared in this way were determined using immuno-electrophoresis. A third batch of IgG, which has been previously prepared in the laboratory, was also tested for comparison.

This technique was carried out using glass photographic plates coated with 2% w/v agar in barbitone buffer containing 0.1% w/v sodium azide. A sample of each batch of IgG, together with a sample of human serum were applied to the plates which were then placed in a bath filled with sodium barbitone/acetate buffer pH 8.6. It was electrophoresed for approximately 3 hours at 10mA 100V. Bromophenol blue was used as a migration indicator.

After this time, anti-human IgG was added to the troughs of one plate and anti-whole human serum added to the troughs of the second plate. The plates were then placed in a humid container and incubated at room temperature overnight. The plates were then washed three times in 0.9% w/v saline and, after drying, were stained with 0.1% w/v Ponceau S in 3% w/v trichloroacetic acid and destained in 5% v/v acetic acid. The plates were photocopied and the photocopy was traced (Figure 2.2).

Aggregation of IgG

Suitable aliquots of IgG were placed in a water bath at 63°C for 30 minutes. At the end of this time, aggregates had formed which sedimented rapidly on standing. Aggregates were always formed just prior to each experiment and were never stored.

2b) n-Formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP)

10mg of FMLP were dissolved in 1ml dimethyl sulphoxide and adjusted to a final concentration of 10mM with CMFSS. This was stored at -70°C in 100µl aliquots and appropriate dilutions made with CMFSS as required.

2c) Opsonized Zymosan

40mg of Zymosan A were boiled for 30 minutes in 0.9% w/v saline + 2mM calcium chloride. The zymosan was washed once by centrifugation in 0.9% w/v saline + 2mM calcium chloride and the pellet resuspended in 8ml of fresh human serum. After incubation at 37°C for 30 minutes, the coated zymosan was washed twice with PBS and suspended at a final concentration of 40mg/ml in PBS. Boiled and washed, but otherwise untreated, zymosan A at an identical concentration in PBS was also prepared for comparative purposes. The opsonized zymosan was stored at -70°C for periods up to 14 days.

Plate 1: AGAINST ANTI-HUMAN IgG

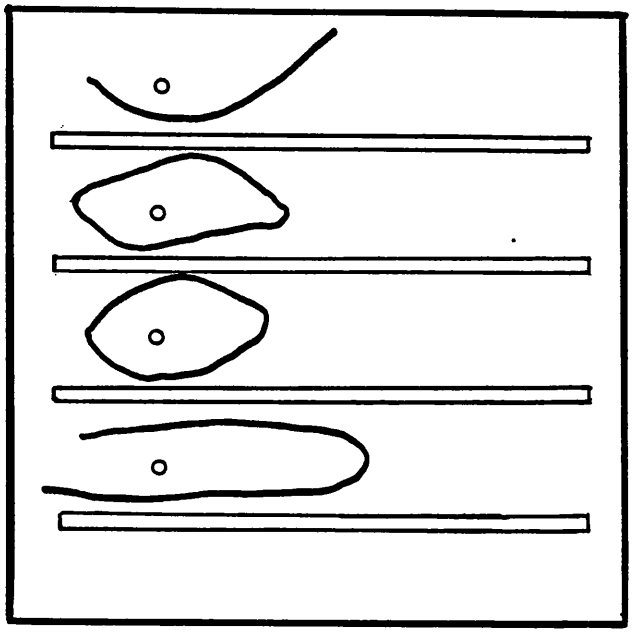
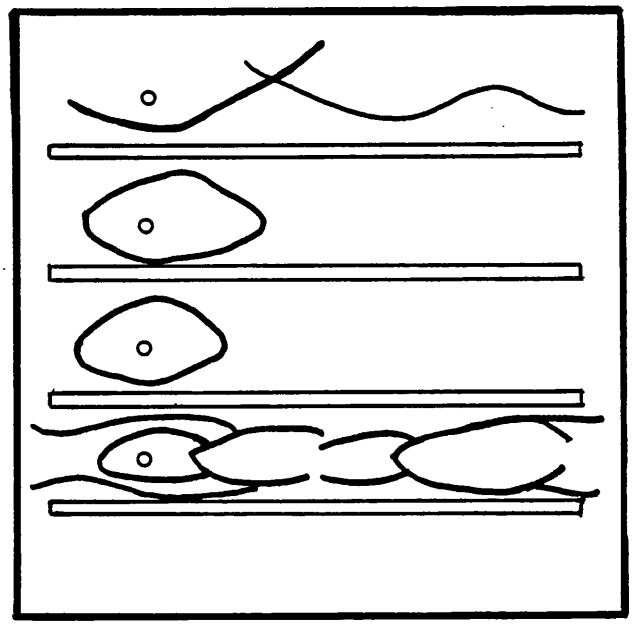


Plate 2: AGAINST ANTI-WHOLE HUMAN SERUM



WELL 1: IgG Batch 1

WELL 2: Laboratory stock IgG

WELL 3: IgG Batch 2

WELL 4: Human Serum

Figure 2.2 IMMUNO-ELECTROPHORESIS PLATES

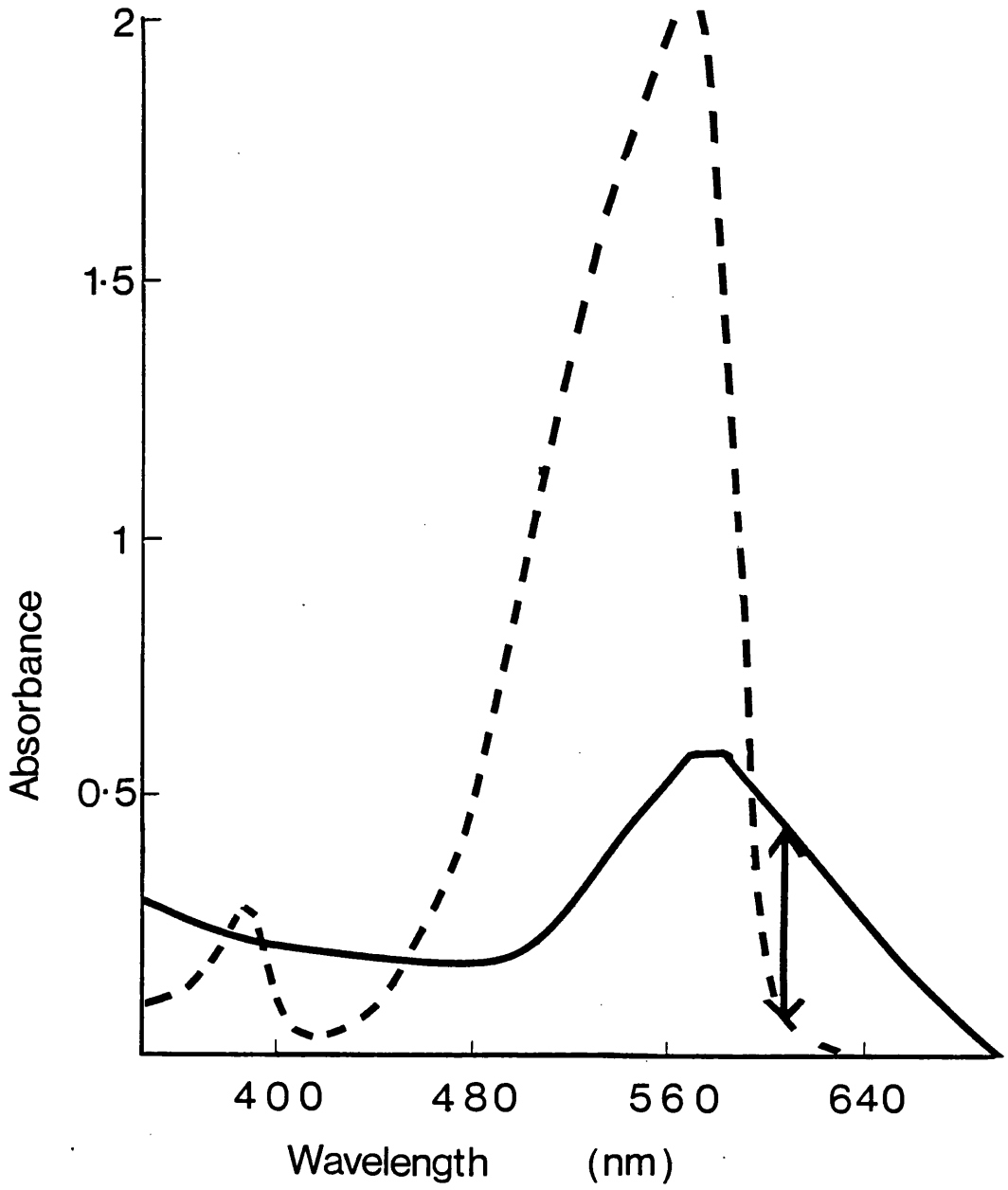
3. ASSAYS FOR HYDROGEN PEROXIDE

3a) OXIDATION OF PHENOL RED

This assay to determine levels of hydrogen peroxide generated by phagocytic cells was first described by Pick and Keisari (1980, 1981). The assay is based on the hydrogen peroxide - dependent, horseradish peroxidase - mediated oxidation of phenol red resulting in the appearance of a compound with an increased absorbance at 610nm at alkaline pH from that of native phenol red (Figure 2.3).

Standardization of Assay

First the phenol red assay was standardized by using hydrogen peroxide solutions in distilled water of known concentrations. To 1ml aliquots of buffered phenol red solution in LP3 tubes, 10µl of appropriate dilutions of hydrogen peroxide were added to result in a range of final concentrations of hydrogen peroxide from 1µM - 100µM. The tubes were incubated for 5 minutes at room temperature, 10µl of 3M NaOH added to raise the pH to 12.5 and the absorbance at 610nm read in the spectrophotometer against blanks containing 1ml buffered phenol red solution, 10µl 3M NaOH and 10µl hydrogen peroxide at the corresponding dilutions (added after the 3M NaOH). It was established that there was a linear relationship between absorbance at 610nm and hydrogen peroxide concentration in the 1-60µM range, corresponding to 1-60nmoles hydrogen peroxide per ml. This procedure was repeated on several occasions and the results used to compute a 'line of best fit'. This was stored on disc so that accurate, reproducible determinations of concentrations from corresponding absorbance readings could be made at any time (Figure 2.4).



——— H_2O_2(1mM) + Phenol red (20mg/l) + HRPO (50µg/ml) at pH 12.5

– – – Phenol red (20mg/ml) + HRPO (50µg/ml) at pH 12.5

Figure 2.3 ABSORPTION SPECTRA OF NATIVE AND H_2O_2-OXIDIZED PHENOL RED. (FROM PICK AND KEISARI, 1980).

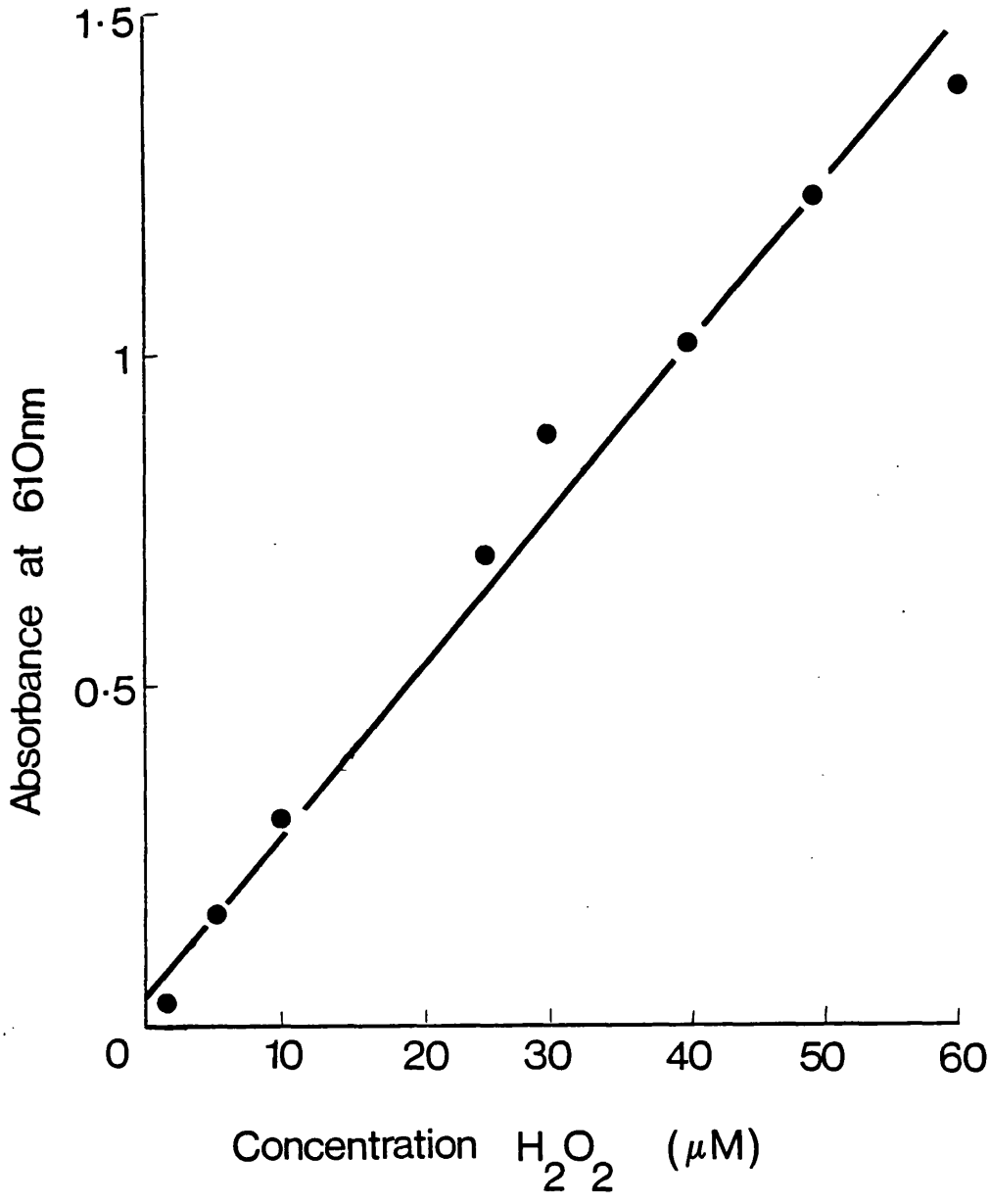


Figure 2.4 CURVE FOR STANDARDIZING THE PHENOL RED ASSAY.

The Use of the Phenol Red Assay for the Quantitation of Hydrogen Peroxide Produced by Phagocytic Cells

Neutrophils or monocytes were suspended, after counting, in buffered phenol red solution at a final concentration of 1 x 10^6/ml or 2 x 10^6/ml as appropriate. The cells were aliquoted in 1ml quantities into LP3 tubes, which already contained any appropriate stimulant, scavenger or inhibitor. The tubes were then incubated at 37°C for the appropriate length of time (usually 30 minutes). After this time the tubes were placed on ice for 5 minutes and then centrifuged at 1500g for 5 minutes. The supernatants were then placed in fresh LP3 tubes containing 10µl 3M NaOH, and the absorbance at 610nm read against corresponding blanks containing 1ml buffered phenol red solution, 10µl 3M NaOH and any appropriate stimulant, scavenger or inhibitor. Each incubation was performed in duplicate and each incubation was run in parallel with duplicates containing 1500 Units catalase. The purple-mauve colour produced on the addition of 3M NaOH was stable for several hours but the results were always read immediately.

3b) FERRITHIOCYANATE ASSAY

This assay was an adaptation of the method described by Thurman, Ley and Scholz (1972), involving the production of a red-brown complex by ferrous ammonium sulphate and potassium ferrithiocyanate in the presence of hydrogen peroxide, under acidic conditions.

Standardization of Assay

To 900µl aliquots of CMFSS in LP3 tubes were added 10µl of appropriate dilutions of hydrogen peroxide in distilled water to result in a concentration range of 5-50µM. To each tube was added 100µl trichloroacetic acid 6M, 200µl ferrous ammonium sulphate 10mM and finally 100µl potassium ferrithiocyanate 2.5M The absorbance at 480nm

was read against a blank containing all those constituents except hydrogen peroxide, which was replaced with 10µl distilled water. A linear relationship was established between the absorbance at 480nm and hydrogen peroxide concentration in the 5-50µM range, corresponding to 5-50nmoles hydrogen peroxide per ml. This procedure was repeated on several occasions and the results used to compute a 'line of best fit'. This was stored in the same way as that for the phenol red assay (Figure 2.5).

The Use of the Ferrithiocyanate Assay for the Quantitation of Hydrogen Peroxide Produced by Neutrophils

Neutrophils were suspended, after counting, in CMFSS at a concentration of 2×10^6/900µl. The cells were aliquoted in 900µl quantities into LP3 tubes already containing any appropriate stimulant, scavenger or inhibitor. The tubes were then incubated at 37°C for 30 minutes. After this time the tubes were placed on ice for 5 minutes, and then centrifuged at 1500g for 5 minutes. The supernatants were then placed in fresh LP3 tubes and 100µl trichloroacetic acid 6M were added to each tube, followed by 200µl ferrous ammonium sulphate 10mM and 100µl potassium ferrithiocyanate 2.5M. The absorbances at 480nm were read immediately against a blank which contained 900µl CMFSS in place of the cell supernatant.

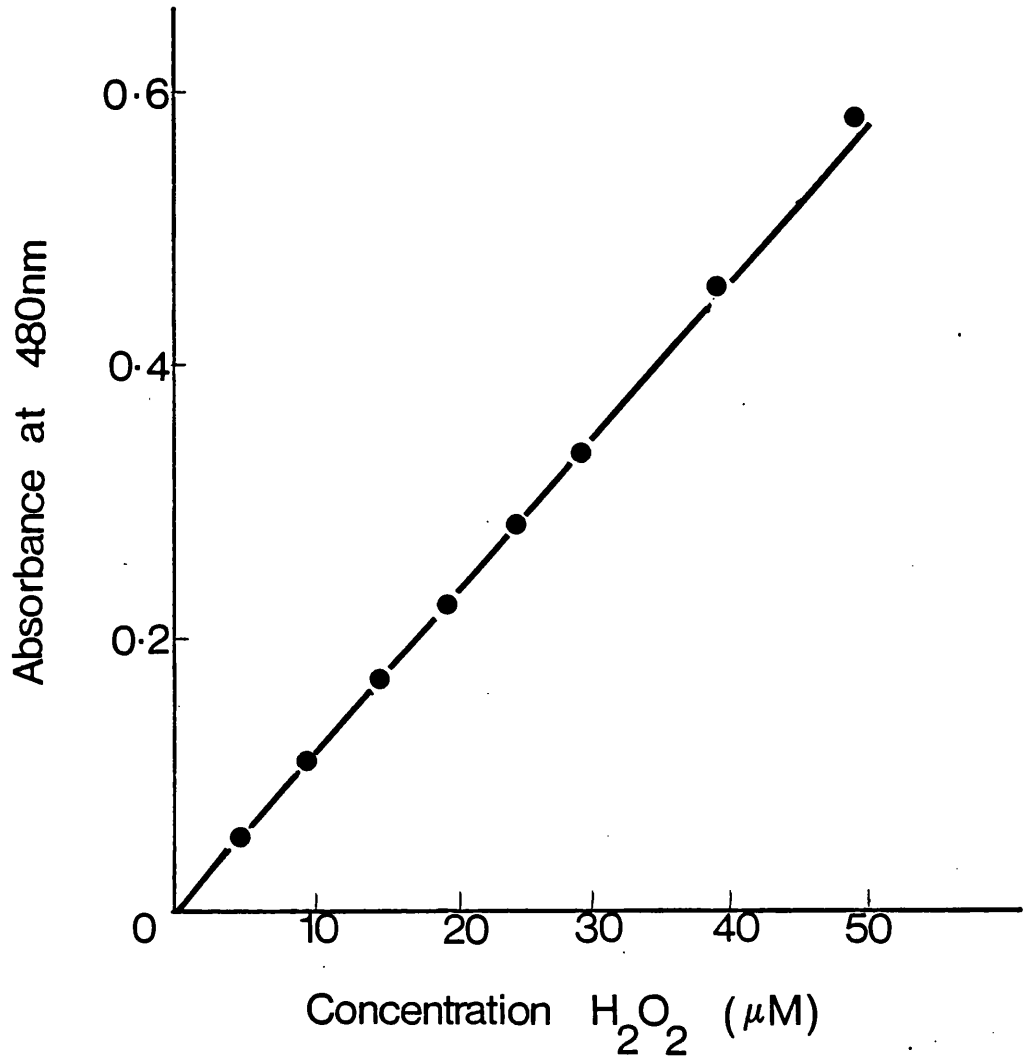


Figure 2.5 CURVE FOR STANDARDIZING THE FERRITHIOCYANATE ASSAY.

4. ASSAY FOR SUPEROXIDE ANION

The assay used was a modification of the micro-assay described by Pick and Mizel (1981) which utilizes the reduction of cytochrome C as the basis for the detection of superoxide anion. Incubations are performed in a flat-bottomed microwell plate and results read using an automatic plate reader which enables large amounts of data to be generated relatively quickly.

4a) Enzymatic Generation of Superoxide Anion

Superoxide anion was generated enzymatically using the xanthine/ xanthine oxidase system to test any effect the different drugs/ inhibitors might have on superoxide anion itself.

A saturated solution of xanthine was made in distilled water and left overnight. This was then filtered immediately prior to use and adjusted to pH 7 with 1M NaOH. 100µl were placed in each well of a 96-well flat-bottomed microtitre plate. The wells already contained the appropriate concentrations of the drugs to be tested in 10µl quantities. Control wells contained 10µl solvent. Superoxide dismutase (50 Units in 10µl CMFSS) was added to certain other control wells. To all wells was added 100µl cytochrome C solution in CMFSS such that the final concentration was 160µM. To start the reaction 10µl xanthine oxidase (5 Units/0.3ml diluted 1 in 25 with CMFSS) was added to each well except the blank and to certain control wells containing just drug. The plate was incubated at room temperature for 10 minutes and then the absorbances at 550nm read using an automatic elisa plate reader.

Each incubation was performed in quadruplicate. The absorbance expressed was the difference between that produced in the absence of superoxide dismutase and that produced in its presence to give the increase in absorbance specific to superoxide.

4b) Measurement of Superoxide Anion Production by Neutrophils

A 96-well microtitre plate was prepared by placing in it appropriate stimuli at various concentrations. Each concentration of each stimuli was performed in quadruplicate. Control wells contained 50 Units superoxide dismutase. After counting, neutrophils were suspended in 160μM cytochrome C at a concentration of 1×10^6/ml or 2×10^6/ml. 200μl aliquots (ie. 2×10^5 or 4×10^5 cells) were placed in the wells and the plate was incubated at 37°C for the appropriate length of time. The absorbances at 550nm were read against a blank containing no cells using an automatic elisa plate reader. The absorbance expressed was the difference between that produced in the absence of superoxide dismutase and that produced in its presence to give the increase in absorbance specific to superoxide.

Calculation of Results

Results were expressed in terms of nmoles cytochrome C reduced using the formula:

$A = Ecl$ A = Absorbance

c = concentration (mM)

l = length of light path (cm)

E = Extinction coefficient = $21.1 mM^{-1} cm^{-1}$

l was determined by measuring the radius (r) of the wells and then applying the formula:

$$\text{Volume} = \Pi r^2 h$$

where h is the height of liquid in the well and therefore the length of the light path.

5. OXYGEN CONSUMPTION

Oxygen uptake by neutrophils was measured using a Clark-type oxygen electrode maintained at 37°C. This electrode incorporated a magnetic stirrer and was used in conjunction with a potentiating and zero suppress unit and a chart recorder.

5a) Calibration of Electrode

The electrode was allowed to equilibrate with 2ml CMFSS at 37°C inside the reaction chamber. When a stable trace was attained (the "air line") a few crystals of sodium dithionite were added to the reaction chamber. The output dropped rapidly as the dithionite reacted with the dissolved oxygen. The new value attained at the lower output was known as the "N_2 line". The difference between the air-line and the N_2 line represents the oxygen concentration which, for 2ml CMFSS at 37°C is equal to 360nmoles oxygen (Hansatech literature). Once the electrode was calibrated in this way, the scale could be linearly expanded by altering the mV setting of the recorder to increase the sensitivity.
The reaction chamber was then washed several times with more CMFSS to remove all traces of dithionite.

5b) Oxygen Uptake by Neutrophils

After counting, neutrophils were suspended at a concentration of 1×10^6/900µl in CMFSS. 1.8ml of cell suspension was introduced into the reaction chamber and allowed to equilibrate until a steady trace was attained. Then 200µl of the appropriate stimulus was introduced into the reaction chamber through the plunger, by means of a micro-syringe. With the chart recorder set at a known speed, the consumption of oxygen by the cells in response to the stimulus could

be monitored over a set time period. The chamber was washed several times with CMFSS between each incubation.

In practice this method was not used as extensively as it had been hoped. Due to the length of equilibrating times, washing times and only one set of conditions prevailing at any one time, very few results could be gathered before the cells began to aggregate or die.

6. MEASUREMENT OF SERUM SULPHYDRYL LEVELS

Levels of serum sulphydryl (SH) groups can be measured using Ellmann's Reagent (DTNB 0.8mg/ml). Serum SH groups can be oxidized by hydrogen peroxide and consequently the lowered levels of residual SH groups can be determined by this method. (Hall, Blake and Bacon 1982; Hall, Maslen and Blake 1984).

6a) Oxidation of Serum SH Groups by Hydrogen Peroxide

SH oxidation mediated by hydrogen peroxide was assessed by reacting 50µl aliquots of serum with various concentrations of hydrogen peroxide diluted in CMFSS. Each mixture (700µl) was incubated at 37°C for 30 minutes after which the reaction was terminated by the addition of 1500 Units catalase contained in 100µl CMFSS. SH levels were determined as outlined below.

6b) Oxidation of Serum SH by Hydrogen Peroxide Produced by Stimulated Neutrophils

Each incubation in LP3 tubes contained an appropriate number of neutrophils, 4mg heat-aggregated IgG and 100µl serum in a final volume of 2ml. Certain control incubations contained 1500 Units catalase. The cells were incubated for 30 minutes at 37°C and the reaction terminated by the addition of catalase (1500 Units). The tubes were

centrifuged for 5 minutes at 750g and residual serum SH levels were determined as outlined below.

6c) SH Assay

800µl of hydrogen peroxide + serum system or 800µl of cell supernatant were incubated with 200µl DTNB (0.8mg/ml in 0.1M phosphate buffer pH 7.4) for 5 minutes at 37°C. The absorbance at 440nm was then read against a blank containing CMFSS in place of the supernatant and DTNB. The reading obtained was converted to serum SH levels in $\mu mol.l^{-1}$ by reference to a calibration curve obtained using reduced glutathione in the assay system.

This assay was also used to determine the effects of drugs on levels of SH groups. In these cases, the incubations contained 50µl serum, 10µl appropriate concentration of drug and 750µl CMFSS. After incubation at 37°C for 30 minutes, 200µl DTNB (0.8mg/ml) were added and the procedure above followed.

7. BLOCKADE OF CELL SURFACE SULPHYDRYL GROUPS

Neutrophils and monocytes were separated as described. After one wash with CMFSS, the two cell populations were divided into half and one half of each cell population was incubated for 1 hour at 37°C with the irreversible, non-penetrating SH blocker parahydroxymercuriphenyl-sulphonic acid (pHMPSA) (Tsan and Berlin, 1971). All cells were then washed three times with CMFSS and, after counting, resuspended in buffered phenol red solution at a concentration of 1×10^6/ml. The cells were then stimulated and levels of hydrogen peroxide produced were measured as described.

8. EA ROSETTE ASSAY

8a) Preparation of Antibody by Affinity Chromatography

The IgG fraction used to sensitize calf erythrocytes was prepared from a rabbit anti-calf erythrocyte antiserum by affinity chromatography on a protein A-Sepharose CL-4B column. Protein A binds the Fc portion of IgG (1,2,4) at neutral pH. This interaction is disrupted at low pH and hence the IgG can be recovered using acetic acid. This procedure was based on the method of Hjelm and Hjelm as used by Hall (Hall, 1978).

The protein A-Sepharose CL-4B column was washed with PBS for 10 minutes and then 2ml of rabbit antiserum was added to the column. This was washed through with PBS until the first protein fraction was eluted. This was discarded. 1M acetic acid was then added to the column and the IgG fraction collected on ice in a fresh container. The IgG fraction was concentrated to approximately 10ml using a positive pressure Amicon unit fitted with a PM50 membrane. The concentration of the protein solution was estimated by measuring its absorbance at 280nm and 260nm and by reference to a nomogram. The IgG solution was dialysed against PBS at 4°C for 4 hours, with a change of PBS after 2 hours. The IgG was stored at -20°C in 200µl aliquots and thawed only once before use.

8b) Detection of Neutrophil Fc Receptors

Calf erythrocytes in Alsevers solution were washed three times with CMFSS. They were then resuspended at 2% v/v in CMFSS and sensitized by incubation with an equal volume of appropriate dilutions of the prepared antibody at 37°C for 30 minutes. The erythrocytes were then washed three times in CMFSS and resuspended in CMFSS at 1% v/v.

Neutrophils were prepared as previously described and resuspended at a concentration of 2 x 10^6/ml in CMFSS. 200µl of neutrophil suspension were mixed with 200µl of each sensitized erythrocyte suspension in LP4 tubes. The tubes were then centrifuged at 110g for 3 minutes and incubated on ice at 4°C for one hour. The pellets were then resuspended on a rotating turntable (20 rpm) for 30 seconds. 50µl of a saturated solution of methyl violet in CMFSS was added to each tube and the number of rosette-forming cells counted using a haemacytometer. A rosette was defined as 3 or more erythrocytes in contact with a neutrophil. At least 100 neutrophils were counted and the percentage of those which had formed rosettes calculated.

9. STATISTICAL ANALYSIS

Unless otherwise stated, statistical significance was tested for by the Mann-Whitney U test for non-parametric data. Correlation coefficients were obtained from the Spearman's Rank Correlation Coefficient for non-parametric data. All statistical methods were performed using the statistics software package supplied with the Apple IIe computer edited by Bruce Land, 1979 (Inter-Stat).

CHAPTER THREE

RESULTS A

RESULTS

MEASUREMENT OF THE RESPIRATORY BURST OF PHAGOCYTIC CELLS

There are several techniques available for evaluating the respiratory burst of phagocytic cells. Oxygen uptake may be measured; the generation of oxygen-derived free radicals $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ can be assessed using a variety of assays; or the increase in glucose oxidation via the HMP shunt can be measured. Although the majority of work in this thesis employed assays for H_2O_2, both oxygen consumption and superoxide generation were briefly examined.

3a) Oxygen Consumption

Experiments were performed using 2 x 10^6 neutrophils isolated from healthy volunteers in 2ml CMFSS at 37°C. HAGG at various concentrations was added after equilibration and oxygen consumption monitored over 10 minutes using the oxygen electrode as described in Chapter 2. The results for the amount of oxygen consumed in 10 minutes in response to various concentrations of HAGG are shown in Figure 3.1 and a typical kinetic study is shown in Figure 3.2. This demonstrates that, following a lag period of approximately 20 seconds, 50% of the response occurs after 1 minute and after 4 minutes, 95% of the response is complete. Unfortunately it became apparent that this method of monitoring the respiratory burst was not very practical. Cells began to aggregate and die in the stock solution before many experiments could be performed and so it was decided not to continue with this technique.

3b) Superoxide Generation

4 x 10^5 neutrophils from healthy volunteers were stimulated with HAGG or FMLP at various concentrations in the presence of cytochrome C in 200µl incubations at 37°C. The results of dose response and time courses

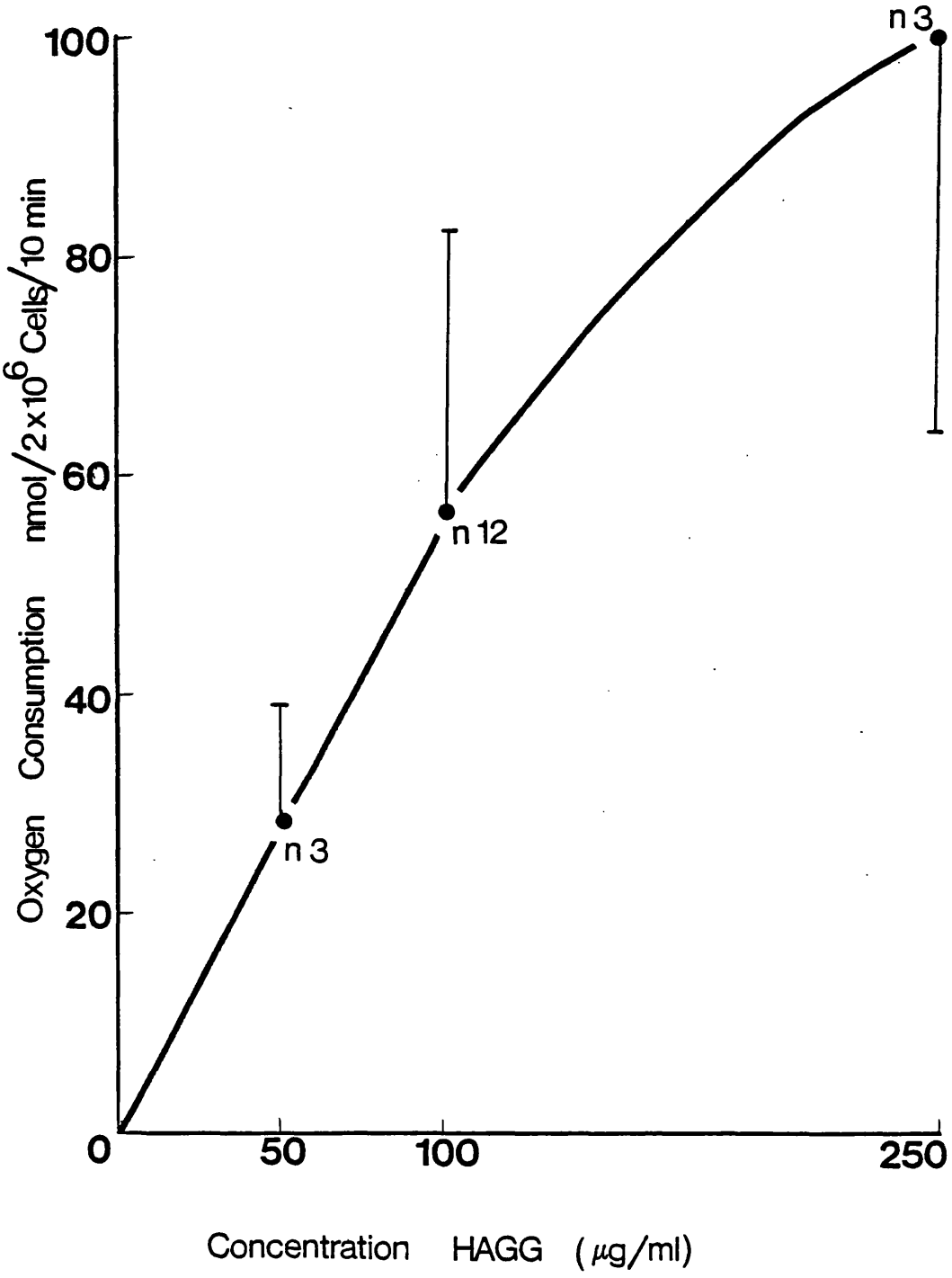


Figure 3.1 Oxygen consumption by human neutrophils stimulated with HAGG after 10 minutes.

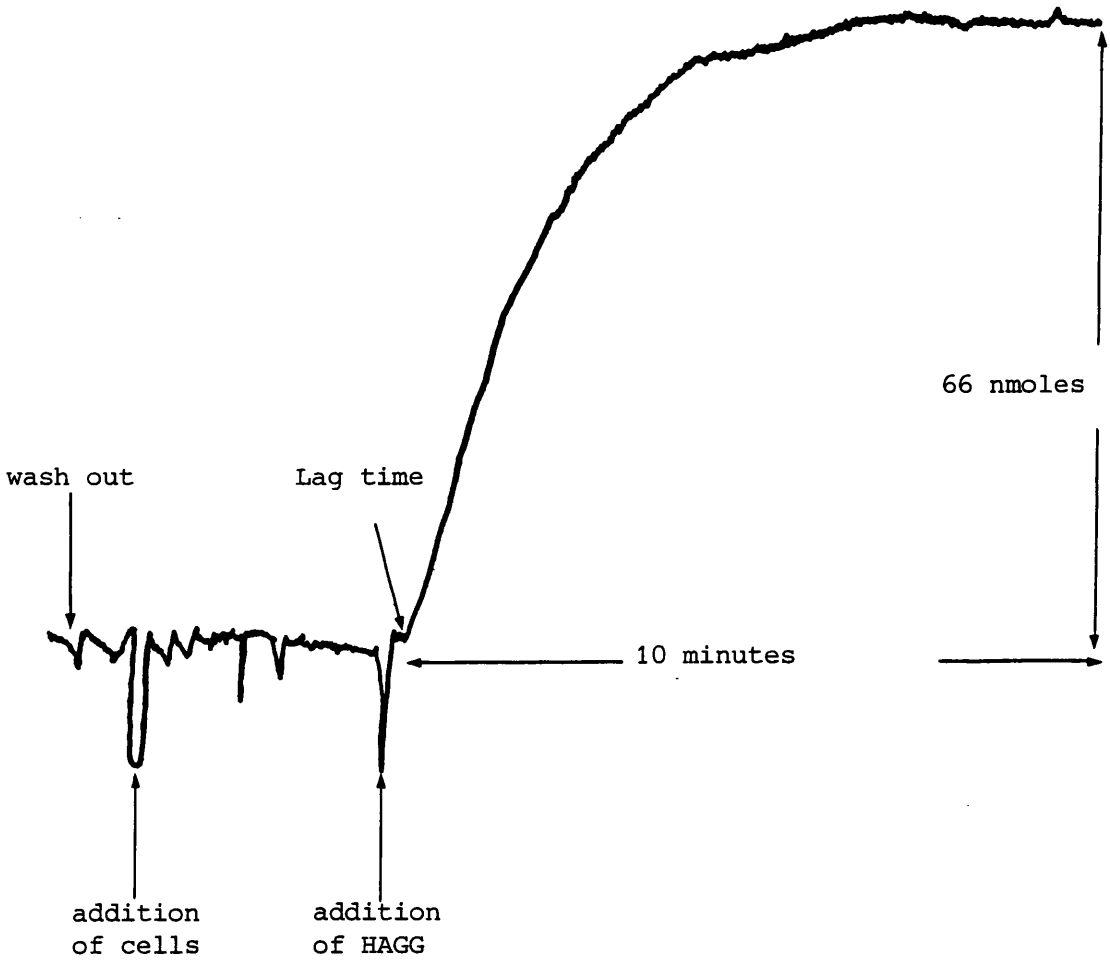


Figure 3.2 Kinetics of O_2 consumption over 10 minutes by 2×10^6 neutrophils stimulated by 200µg HAGG.

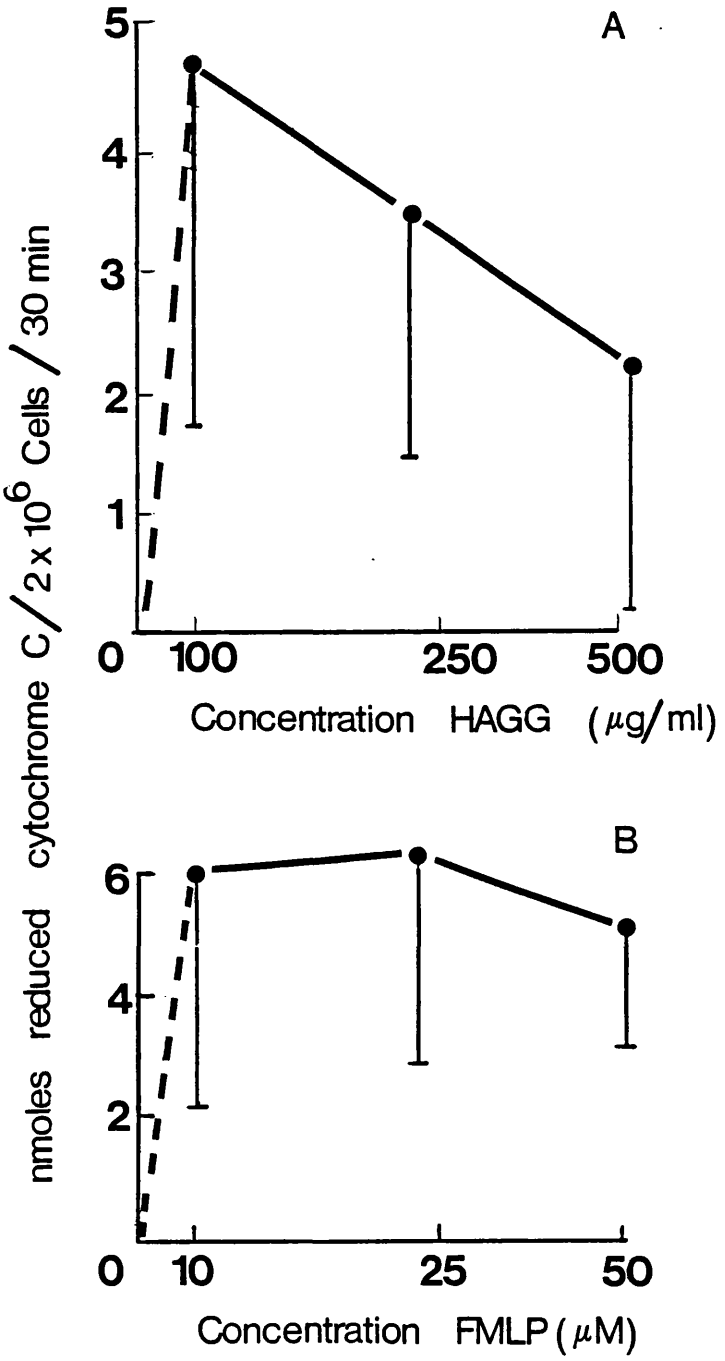


Figure 3.3 Reduction of cytochrome C by stimulated neutrophils after 30 minutes (superoxide specific). Results presented as $\bar{X}$ ± SD of 4 experiments. A:Dose response to HAGG; B:Dose response to FMLP.

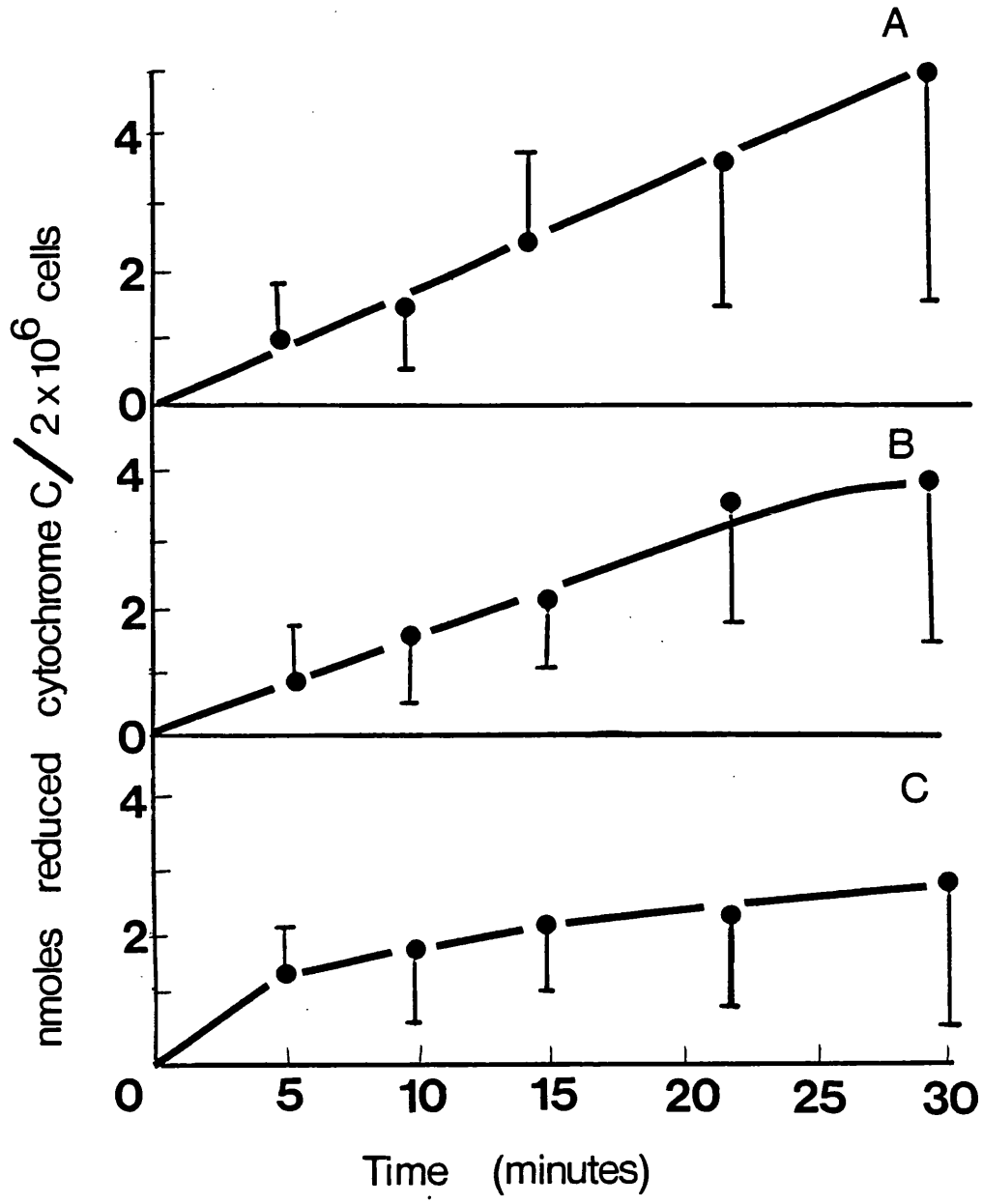


Figure 3.4 Reduction of cytochrome C by neutrophils stimulated by HAGG (superoxide specific). Results presented as $\bar{X}$ ± SD of 4 experiments. A:Time course using 100µg/ml HAGG; B:Time course using 250µg/ml HAGG; C:Time course using 500µg/ml HAGG.

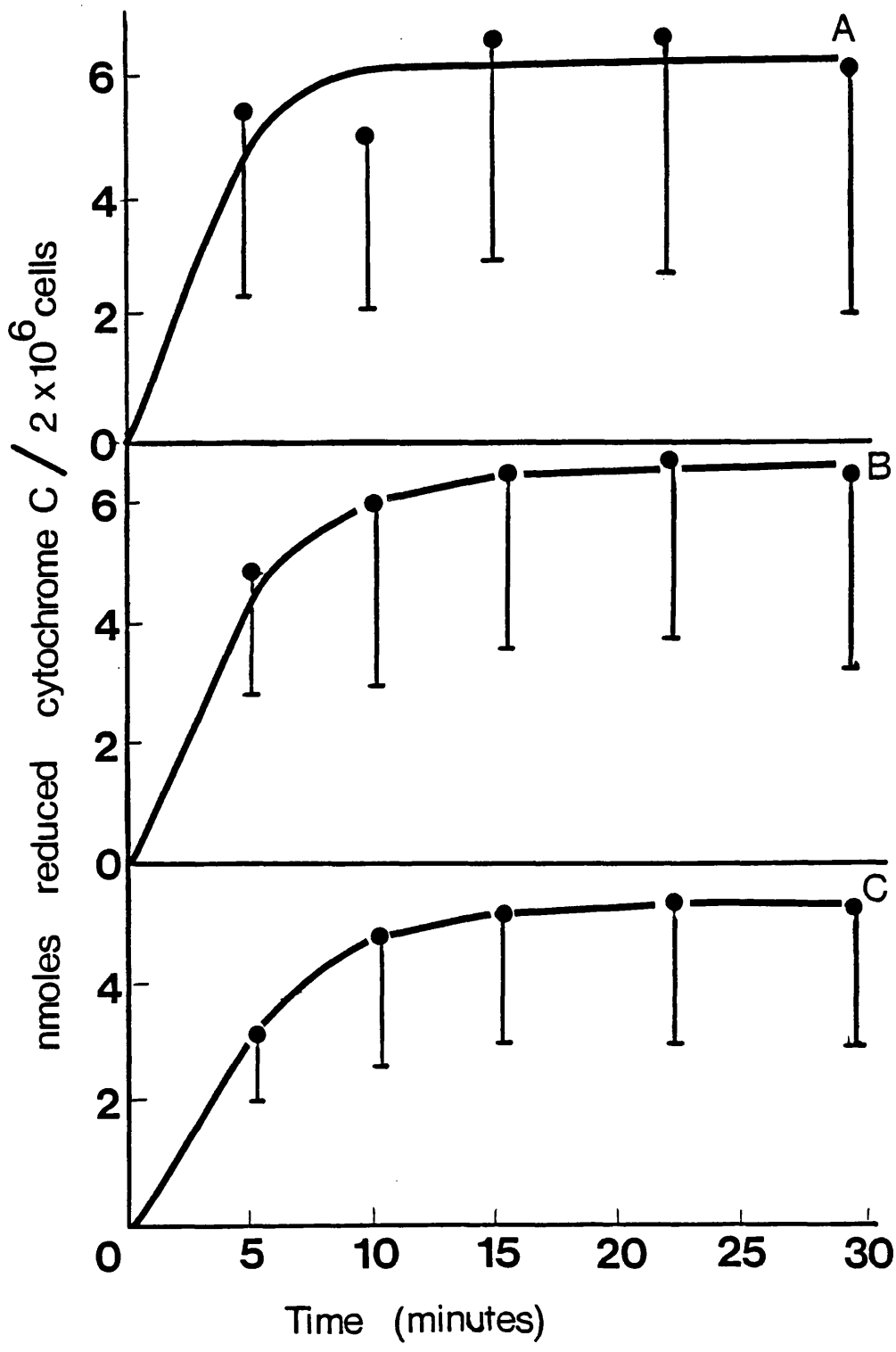


Figure 3.5 Reduction of cytochrome C by neutrophils stimulated by FMLP (superoxide specific). Results presented as $\bar{X}$ ± SD of 4 experiments. A:Time course using FMLP 10μM; B:Time course using FMLP 25μM; C:Time course using FMLP 50μM.

are shown in Figures 3.3 - 3.5. Results are expressed in terms of nmoles of reduced cytochrome C/2 x 10^6 cells. This reduction is superoxide specific i.e. reduction in the absence of superoxide dismutase minus that in its presence. It also takes into account any background release by unstimulated cells. It appears that the higher the concentration of HAGG used the less superoxide is generated and that also the lower concentration of HAGG (100µg/ml) stimulates a steady increase in the generation of superoxide whereas the higher concentration of HAGG (500µg/ml) stimulates an initial generation of superoxide which reduces in rate after 5 minutes.
The concentrations of FMLP chosen (10µM, 25µM, 50µM) appear to stimulate about the same amount of and similar rates of superoxide generation. The time courses for all concentrations show a reduction in rate of generation after 10 minutes.

3c) Peroxide Generation

2 x 10^6 neutrophils from healthy volunteers were incubated with various concentrations of HAGG, FMLP or opsonized zymosan in 1ml incubations in the presence of phenol red and horseradish peroxidase at 37°C. The resulting oxidation of phenol red was expressed in terms of nmoles peroxide as described in Chapter 2.
The results of dose response and time courses are shown in Figures 3.6 - 3.8. All results are corrected for background release.
The results show a dose - and time-dependent generation of peroxide in response to each stimulus. It can be seen that 50% of the maximum response by 2 x 10^6 neutrophils is reached after approximately 4 minutes following stimulation with 200µg/ml HAGG; after about 12 minutes

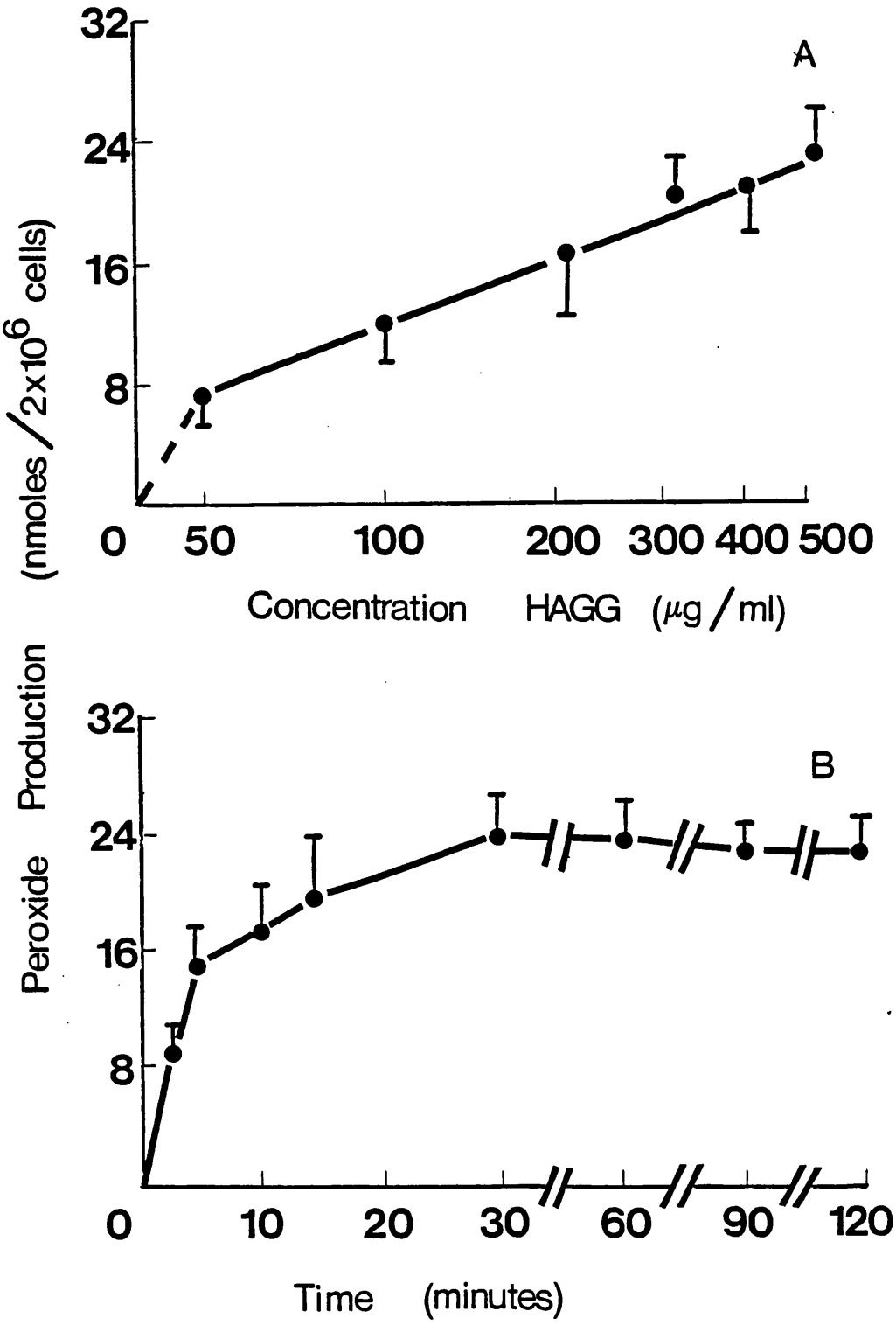


Figure 3.6 Peroxide production by human neutrophils stimulated by HAGG. Results expressed as nmol peroxide produced/ 2×10^6 cells (corrected for background release) and presented as $\bar{X} \pm SD$ of 3 experiments. A:Dose response after 30 minutes; B:Time course using 200µg/ml HAGG.

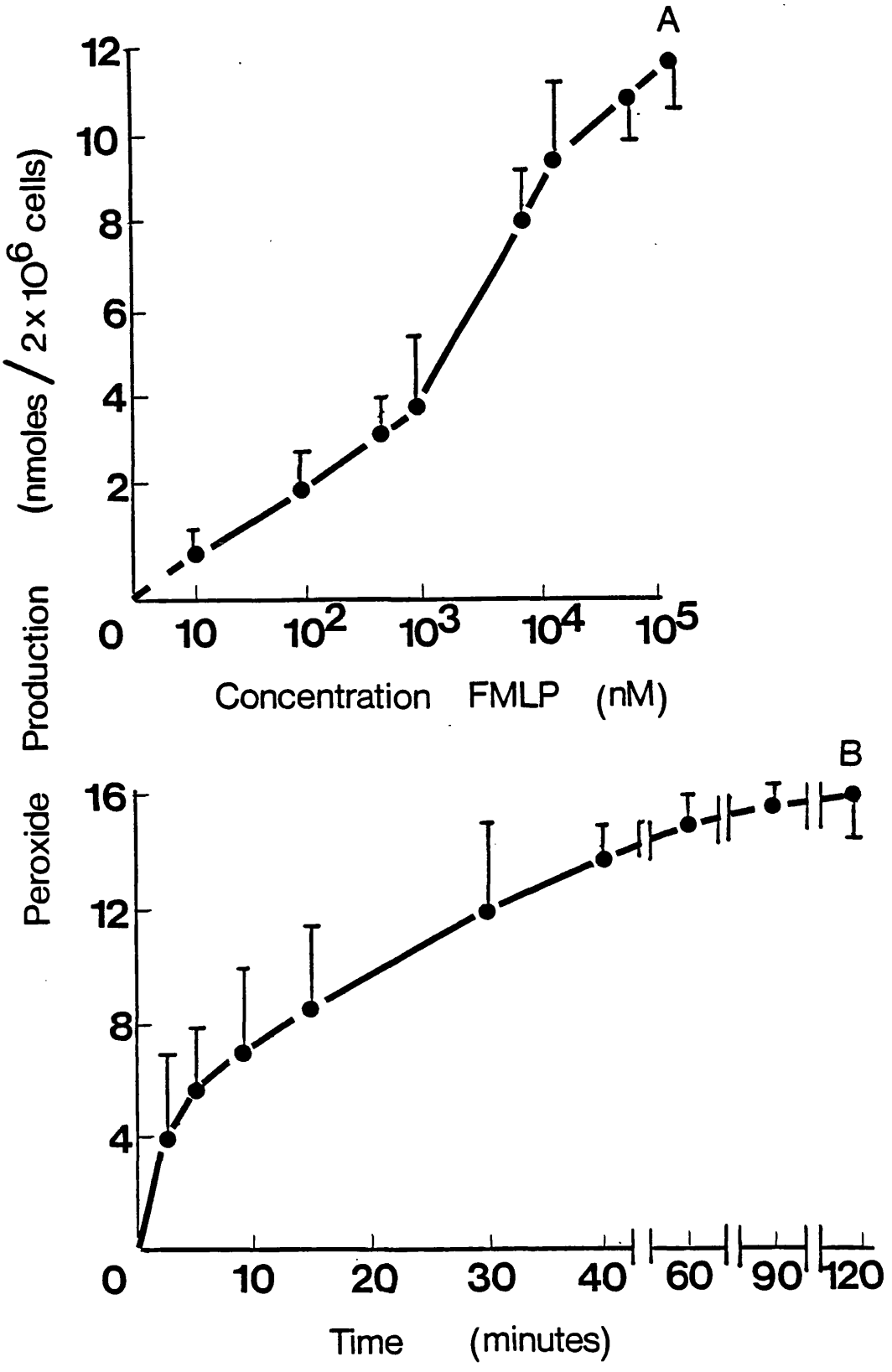


Figure 3.7 Peroxide production by human neutrophils stimulated by FMLP. Results expressed as nmol peroxide produced/ 2 x 10^6 cells (corrected for background release) and presented as $\bar{X}$ ± SD of 3 experiments. A:Dose response after 30 minutes; B:Time course using FMLP 20μM.

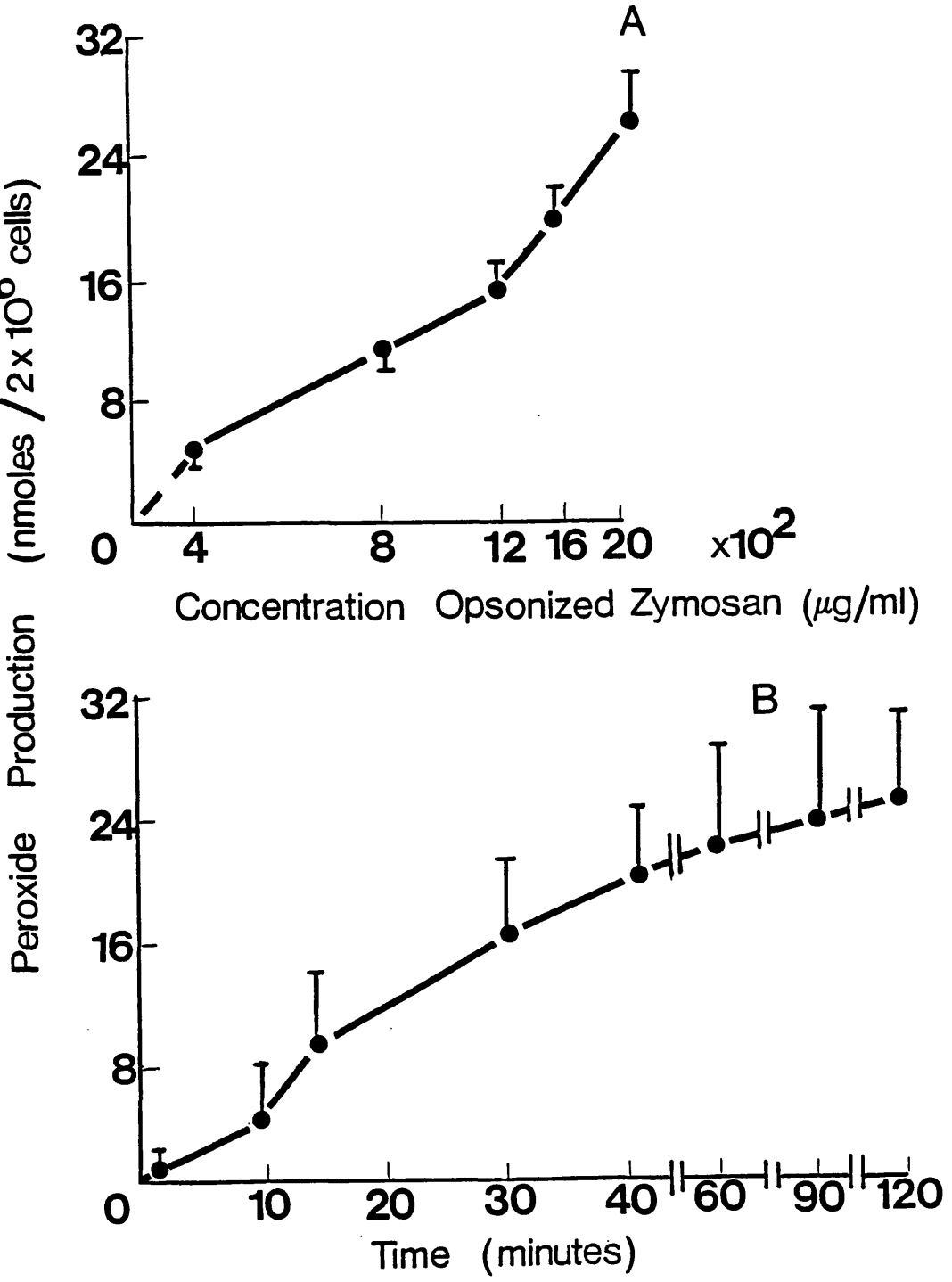


Figure 3.8 Peroxide production by human neutrophils stimulated by opsonized zymosan. Results expressed as nmol peroxide produced/ 2 x 10^6 cells (corrected for background release) and presented as $\bar{X}$ ± SD of 4 experiments. A:Dose response after 30 minutes; B:Time course using 800μg/ml opsonized zymosan.

following stimulation with 20μM FMLP and after about 20 minutes following stimulation with 800μg/ml opsonized zymosan.

As more experiments with these stimuli were performed in the presence and absence of catalase, it quickly became apparent that, according to the stimulus used, not all the oxidation of phenol red could be prevented by this enzyme. Thus it was concluded that not all the oxidation of phenol red was due to hydrogen peroxide (Figure 3.9). These results demonstrate that phenol red oxidation caused by unstimulated or 'resting' cells is 97 ± 6% sensitive to catalase, i.e. it is due almost entirely to hydrogen peroxide. Similarly, cells stimulated by FMLP produce mainly hydrogen peroxide as 93 ± 9% of the oxidation can be inhibited by catalase. However, following stimulation by HAGG, only 23 ± 17% of the phenol red oxidation was inhibited by catalase and the equivalent figure following stimulation by opsonized zymosan was even lower, only 11 ± 9%. Thus cells stimulated by HAGG and opsonized zymosan at these concentrations produce some hydrogen peroxide but mainly another substance which is capable of oxidizing phenol red.

To ensure that sufficient catalase was present to break down any hydrogen peroxide produced various concentrations were added to 1ml aliquots of buffered phenol red solution. To this were added 20 nmoles of hydrogen peroxide and this was then incubated at 37°C for 10 minutes. The results are shown in Table 3.1. The routine concentration of catalase used, 1500 Units/ml, is clearly sufficient to break down any hydrogen peroxide which may be produced and thereby prevent phenol red oxidation.

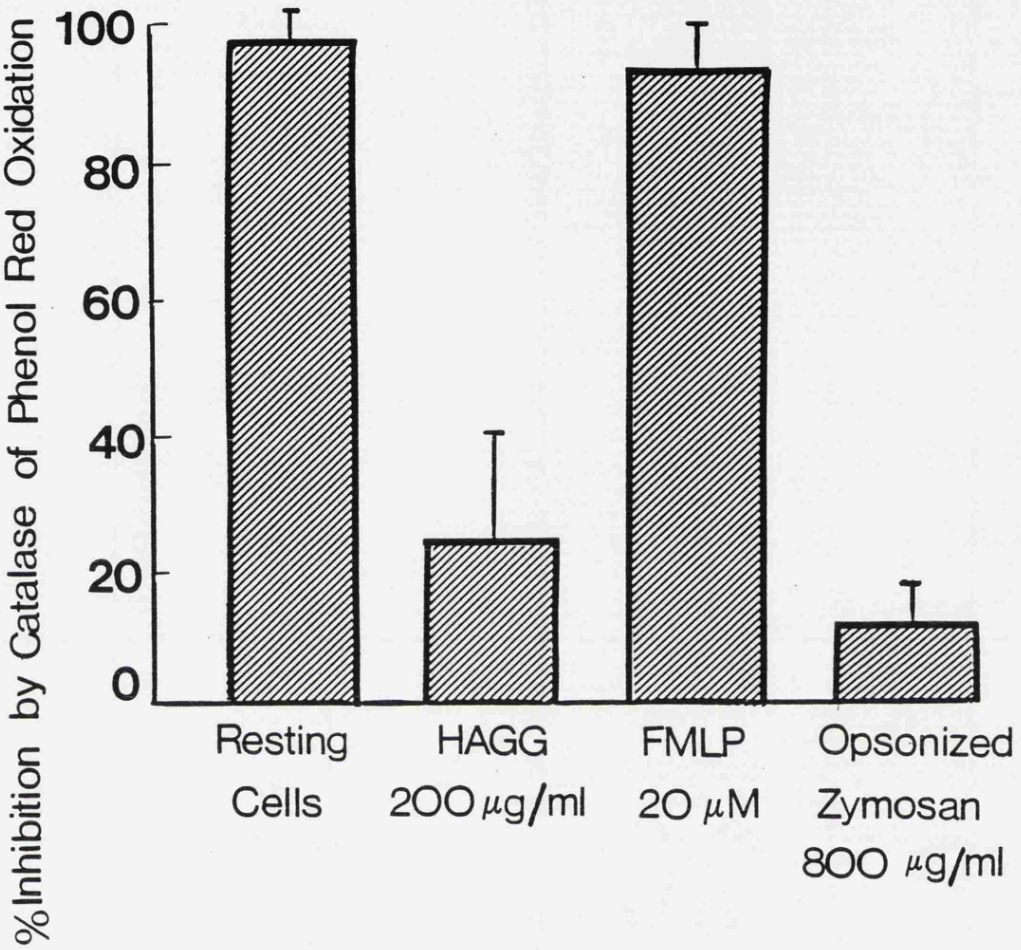


Figure 3.9 % Inhibition by catalase (1500 Units) of phenol red oxidation by 2 x 10^6 neutrophils in response to stimuli and by the cells alone. Results presented $\bar{X}$ ± SD of 10 experiments.

Conc $\frac{n}{}$ Catalase (Units/ml)	Absorbance at 610nm
0	0.500
250	0.018
500	0.005
750	0.016
1000	0.004
1250	0
1500	0
1750	0
2000	0
2500	0

Table 3.1 Absorbance readings at 610nm for phenol red solutions containing 20 nmoles H_2O_2 in the presence and absence of catalase. Each experiment was set up in duplicate.

Reference to the original paper describing this assay (Pick and Keisari, 1981) showed that they too could not abolish totally the oxidation of phenol red by the addition of catalase. They suggested that this may be due to competition between horseradish peroxidase and catalase for hydrogen peroxide. To investigate this a dose response to catalase was performed in the presence of 2×10^6 neutrophils stimulated by 200µg HAGG. The results are shown in Figure 3.10. As there is very little difference in the catalase insensitive portion of phenol red oxidation regardless of the concentration of catalase used, it would seem unlikely that competition between horseradish peroxidase and catalase for hydrogen peroxide is the explanation for these observations. The failure of catalase to suppress all phenol red oxidation under some circumstances could be due to the HAGG and opsonized zymosan binding to or inactivating the enzyme. Thus an assay for catalase was performed in the presence of these stimuli. 0.1ml 30% hydrogen peroxide was added to 50ml 0.05M phosphate buffer pH7. A solution of 50 Units/ml catalase in 0.05M phosphate buffer pH7 was also prepared. 0.1ml of this solution was added to 2.9ml of the hydrogen peroxide solution in a cuvette and the absorbance at 240nm read. The time required for the absorbance to decrease from 0.450 to 0.400 was noted. This corresponds to the decomposition of 3.45µmoles hydrogen peroxide. This was then repeated in the presence of each stimulus in turn. If they were binding or inactivating the catalase then the time taken for the absorbance to drop would be considerably lengthened. The results in Table 3.2 show that this is not the case and thus the stimuli cannot be directly interfering with the ability of catalase to break down hydrogen peroxide.

To check that the catalase insensitive portion of phenol red oxidation was not due to an intra-cellular source of hydrogen peroxide produced in response to HAGG and opsonized zymosan but not FMLP,

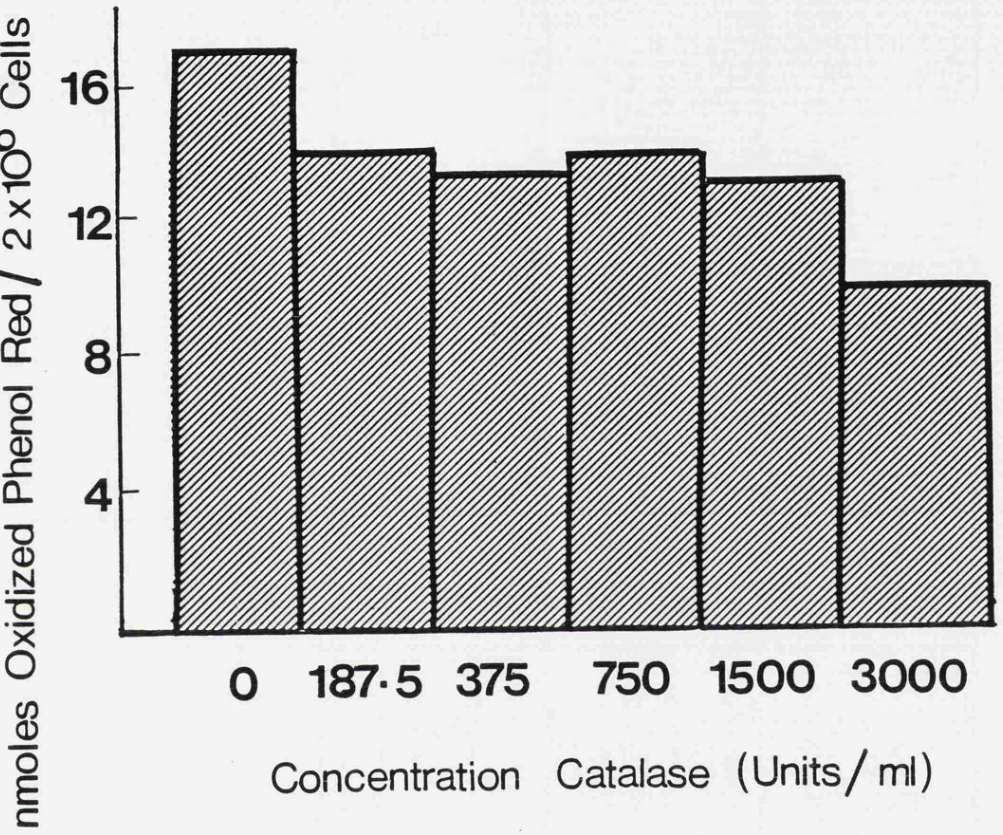


Figure 3.10 Oxidized phenol red produced by 2 x 10^6 neutrophils stimulated by 200µg HAGG in the presence of increasing concentrations of catalase.

Time taken for absorbance at 240nm to drop from 0.450 to 0.400 (seconds):	$\bar{X}$ ±SD n=5
No stimulus	42 ± 3
+ 100µg HAGG	48 ± 8
+ 400µg opsonized zymosan	49 ± 7

Table 3.2. The time taken for 5 Units of catalase to decompose 3.45 µmoles H_2O_2 in the presence and absence of HAGG (100µg) and opsonized zymosan (400µg).

Incubation	nmoles oxidized phenol red/$2x10^6$/30 mins.	
	+ HRPO	- HRPO
Resting cells	7	0
Cells + 200µg HAGG	9	0
Cells + FMLP (20µM)	11	0
Cells + 800µg opsonized zymosan	10	0

Table 3.3. Oxidized phenol red as a result of stimulation of $2x10^6$ neutrophils in the presence and absence of horseradish peroxidase.

experiments were performed where cells were incubated with the stimuli as normal but were then disrupted with alkali and then removed by centrifugation before measuring the absorbance. Parallel incubations had cells removed by centrifugation and the supernatants treated with alkali. There was no difference in the absorbance readings obtained using either method (results not shown).

The assay was also performed with neutrophils stimulated by all three stimuli in the presence and absence of horseradish peroxidase. The results in Table 3.3 show that omission of peroxidase results in total abolition of any oxidation of phenol red. Thus this indicates that whatever this other agent that is capable of oxidizing phenol red might be, it must be a peroxide and is henceforth referred to as ROOH.

To determine whether ROOH is produced at the same rate as any hydrogen peroxide generated in response to a stimulus by neutrophils, a time course was performed following the stimulation of HAGG in the presence and absence of catalase. The results were corrected for any background release of hydrogen peroxide and are shown in Figure 3.11. This demonstrates that there is a lag period associated with the release of ROOH but following that, release progresses at much the same rate as the rate of release of hydrogen peroxide. However, virtually all the hydrogen peroxide production is complete within 10 minutes whereas release of ROOH continues at much the same rate for at least 30 minutes.

The ability of different concentrations of HAGG to promote the release of H_2O_2 + ROOH was also assessed. These data are presented in Figure 3.12 and show that at low concentrations of HAGG, virtually all the phenol red oxidation is due to hydrogen peroxide whereas at high concentrations, it is largely due to ROOH. Figure 3.13 shows the absolute amounts of hydrogen peroxide and ROOH produced in response to different concentrations of HAGG. It can be seen that whilst ROOH

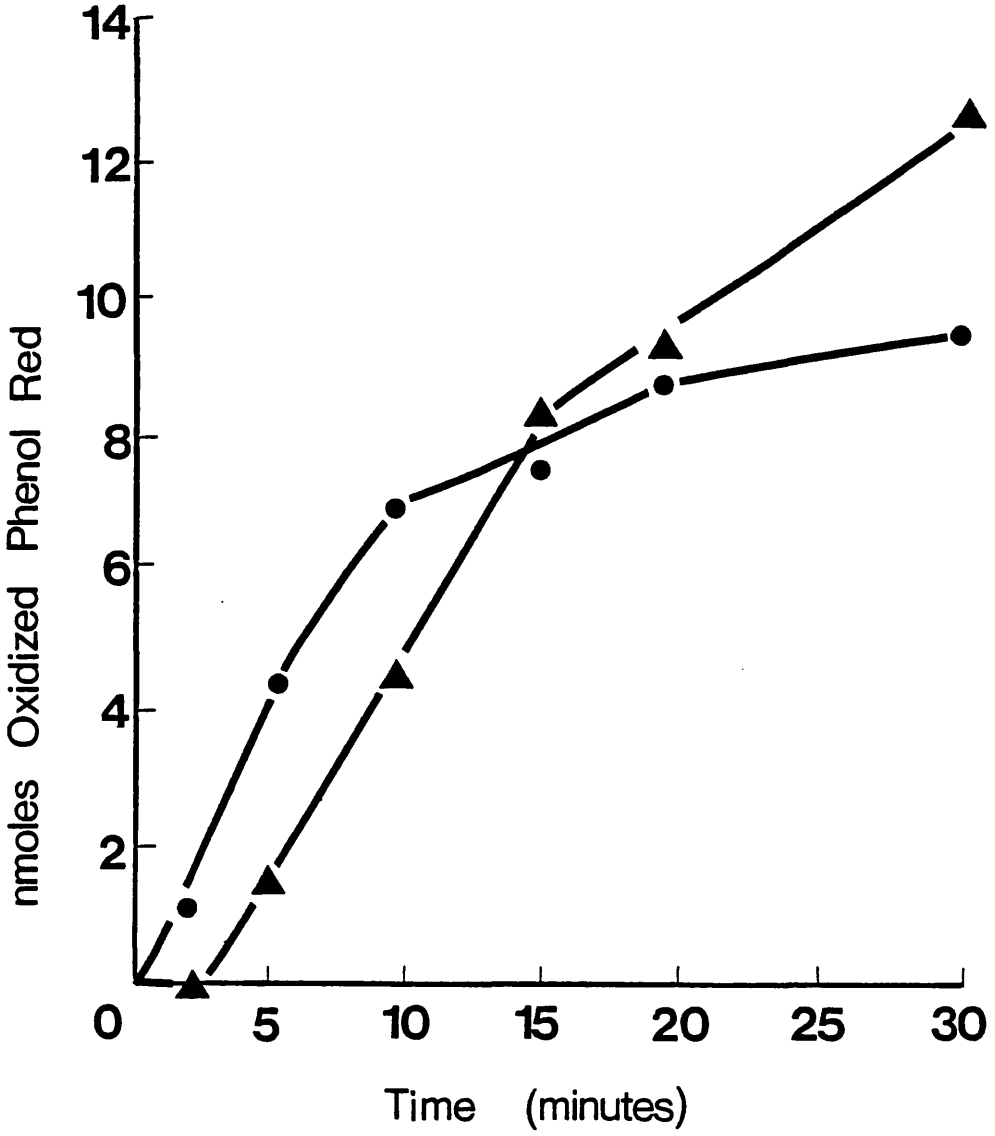


Figure 3.11 Oxidized phenol red in response to 2 x 10^6 neutrophils stimulated with 200µg HAGG over 30 minutes (n=2).

● Oxidation due to H_2O_2

▲ Oxidation due to ROOH

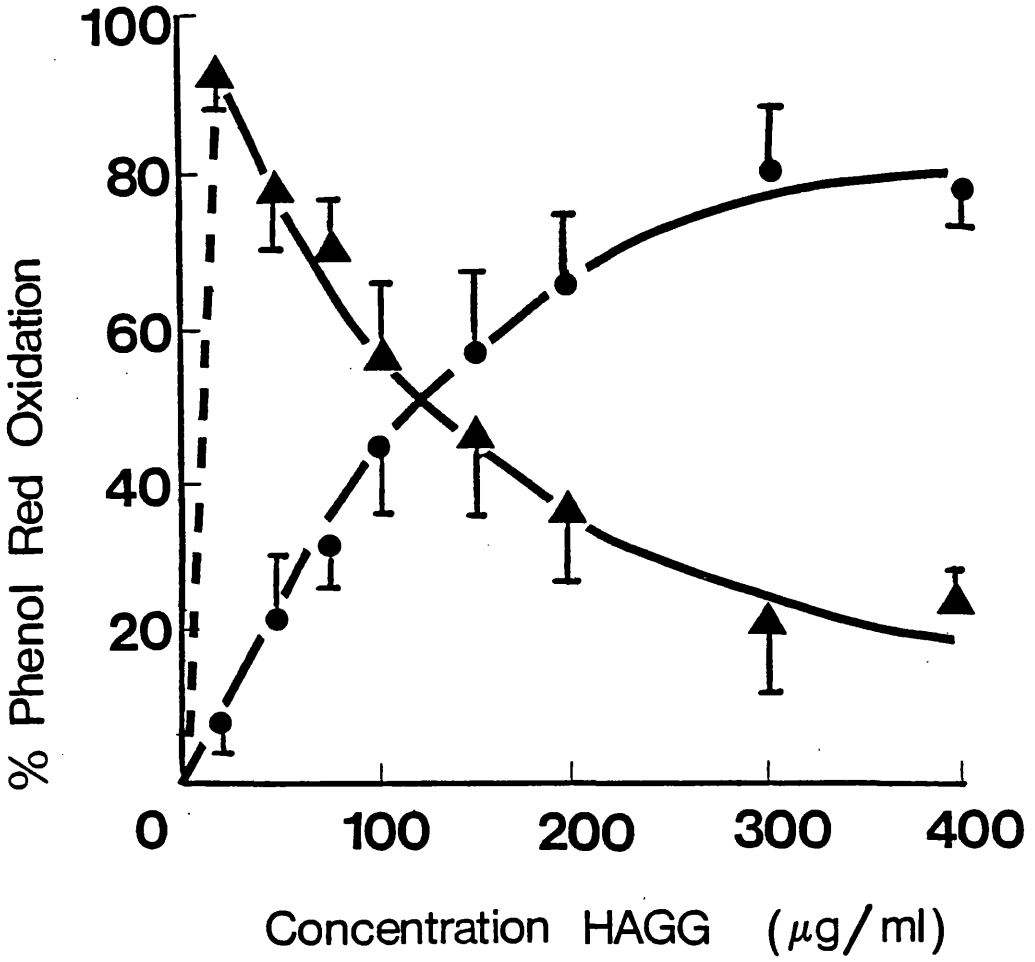


Figure 3.12 % total phenol red oxidation due to ● ROOH and ▲ H_2O_2 following stimulation of 2 x 10^6 neutrophils by various concentrations of HAGG for 30 minutes at 37°C.

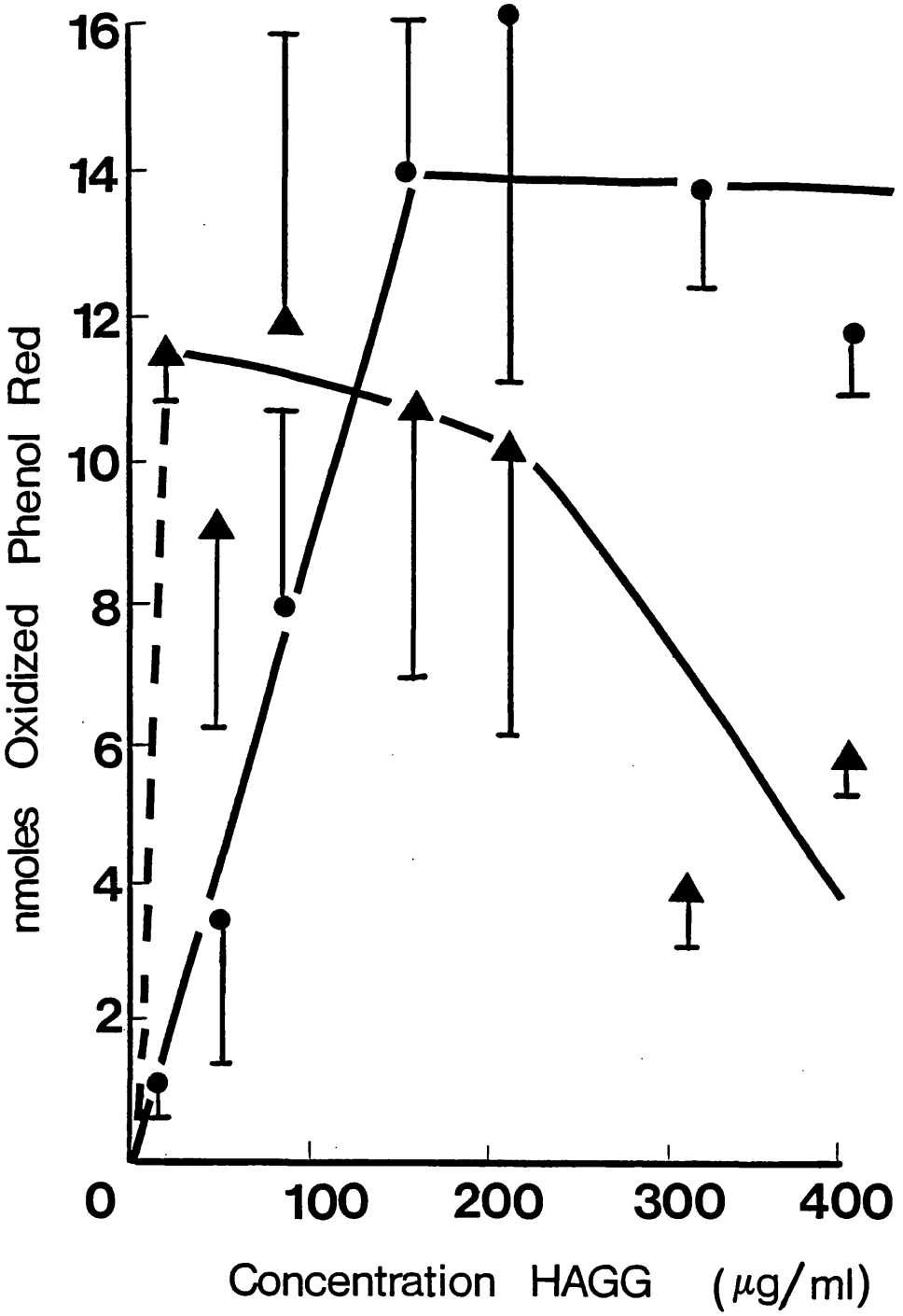


Figure 3.13 Absolute amounts of oxidized phenol red due to ● ROOH and ▲ H_2O_2 following stimulation of 2 x 10^6 neutrophils by various concentrations of HAGG for 30 minutes at 37°C.

production increases with increasing HAGG concentrations, hydrogen peroxide production remains the same, is maximal at the lowest concentrations of HAGG and decreases at higher concentrations.

3d) Oxidation of Serum Sulphydryl Groups by H_2O_2 Released by Stimulated Neutrophils

Serum SH levels are depressed in patients with RA (Lorber et al, 1964). This depression is due to oxidation and subsequent mixed disulphide formation with cysteine of SH groups in albumin (Thomas and Evans, 1975). This SH oxidation could be mediated by hydrogen peroxide and to test this hypothesis this reagent was tested directly in the serum SH assay described in the previous chapter.

The results shown in Figure 3.14 show that hydrogen peroxide oxidises serum SH groups in a dose-dependent manner. Thus it may be calculated that oxidation of 25% of the serum SH groups in this assay system requires the presence of 300-400nmoles H_2O_2. From Figure 3.6 it can be seen that *in vitro* 2×10^6 neutrophils can secrete 24 ± 3nmoles H_2O_2 in response to 200µg HAGG. Thus approximately 30×10^6 cells would have to be stimulated in order to demonstrate 25% serum SH oxidation by H_2O_2 released by those cells. Data from three such large-scale experiments are shown in Table 3.4. The results obtained show very close agreement with the degree of serum SH oxidation predicted and complete protection of the serum SH groups was afforded by the presence of catalase in the assay system.

Since the results of this work has been published (Hall, Maslen and Blake, 1984) it has been realised that in fact 2×10^6 neutrophils do not secrete 24nmoles H_2O_2, but probably more like 6nmoles, so the calculation that 30×10^6 cells would produce 300-400nmoles H_2O_2 is

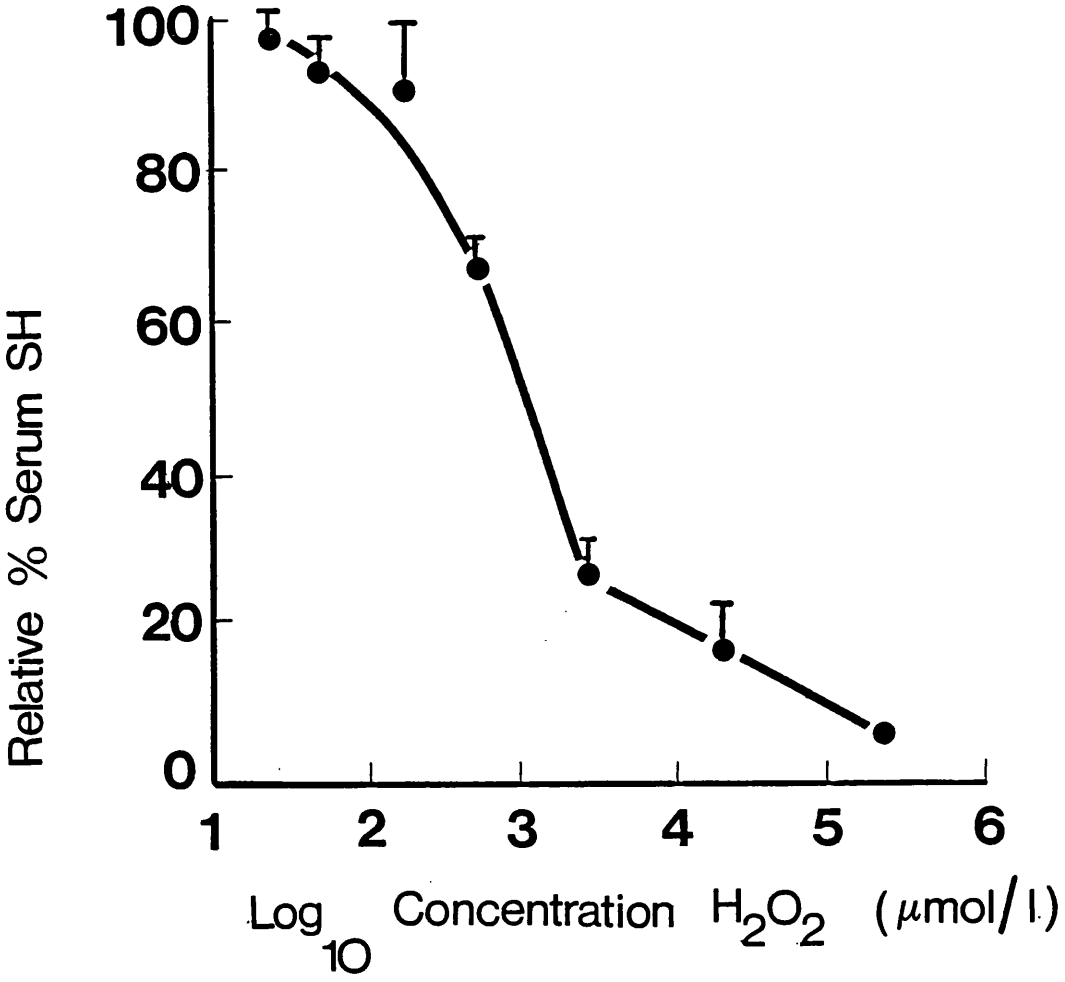


Figure 3.14 Serum SH oxidation by H_2O_2. Results expressed as % serum SH levels relative to values in untreated samples (= 100%). Results presented as $\bar{X}$ ± SD of 3 experiments.

No. of neutrophils per culture x 10^6	Predicted rel. % serum SH	Observed rel. % serum SH	Observed rel. % serum SH + catalase
30	73	73	86
25	78	83	108
32	71	71	99
Mean ± SD		76 ± 5	98 ± 9

Table 3.4 Serum SH oxidation by products of human neutrophils stimulated with heat-aggregated IgG. Each experiment was set up in duplicate with cultures containing neutrophils, 4mg heat-aggregated IgG and 100µl serum in a final volume of 2ml. Catalase (1500 Units) was added to certain tubes as indicated. All incubations were carried out for 30 minutes at 37°C.

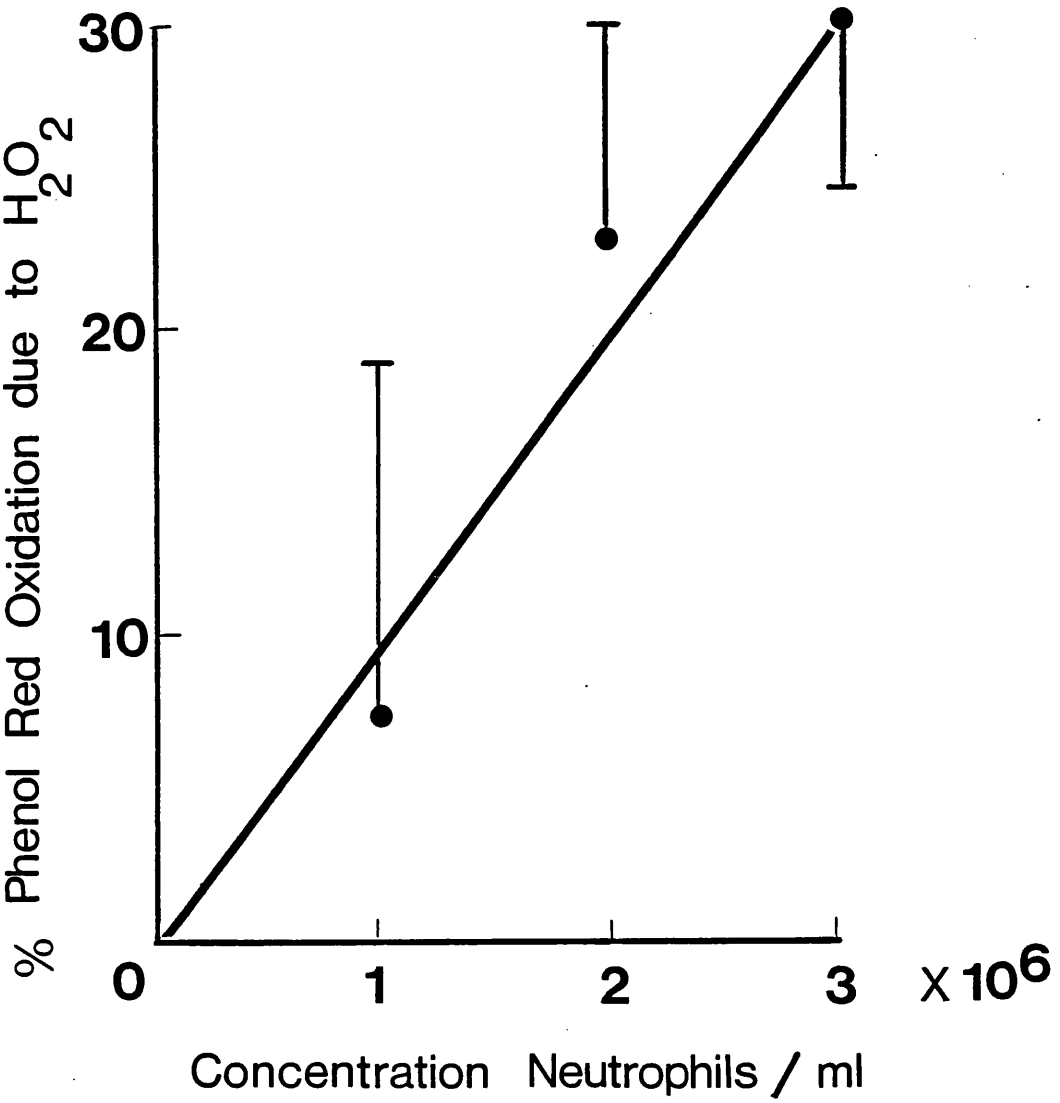


Figure 3.15 % total phenol red oxidation due to H_2O_2 in response to stimulation by 200µg HAGG of increasing concentrations of neutrophils. Results presented as $\bar{X}$ ± SD of 10 experiments.

incorrect. However, this presumes a linear relationship between the number of neutrophils and the amount of H_2O_2 secreted. During the course of this work, using different concentrations of cells, it has become apparent that this is not so. The more cells that are stimulated the proportionally more H_2O_2 is released, and Figure 3.15 demonstrates this. Although it is impossible to measure the amount of hydrogen peroxide produced by 30 x 10^6 cells using the phenol red assay, due to the sensitivity of the assay, it would seem that by extrapolating Figure 3.15, large numbers of neutrophils, when stimulated with HAGG, produce a higher proportion of H_2O_2 than ROOH.

3e) Release of Hydrogen Peroxide From Stimulated Monocytes

Human monocytes were incubated in the phenol red assay with HAGG (200µg/ml), FMLP (20µM) or opsonized zymosan (800µg/ml) at a concentration of 1 x 10^6/ml. Hydrogen peroxide and ROOH release was measured over 30 minutes at 37°C in the presence and absence of catalase. A concentration of 1 x 10^6/ml was chosen because of the lower yields of monocytes compared with neutrophils obtained from human blood.

The results shown in Figure 3.16 demonstrate the much lower levels of hydrogen peroxide (and ROOH) secreted by these cells compared with neutrophils. This is in agreement with work published by Johnston Jr., Lehmeyer and Guthrie (1976); Sagone Jr., King and Metz (1976) and Reiss and Roos (1978). However, Figure 3.17 shows that the proportion of hydrogen peroxide released compared with ROOH is higher from monocytes than neutrophils. The figures are 7% for neutrophils (1 x 10^6/ml) and 62% for monocytes (1 x 10^6/ml) in response to HAGG (200µg/ml). It also seems likely that the proportion of hydrogen peroxide released is greater from monocytes stimulated with opsonized zymosan (800µg/ml) as 1 x 10^6/ml

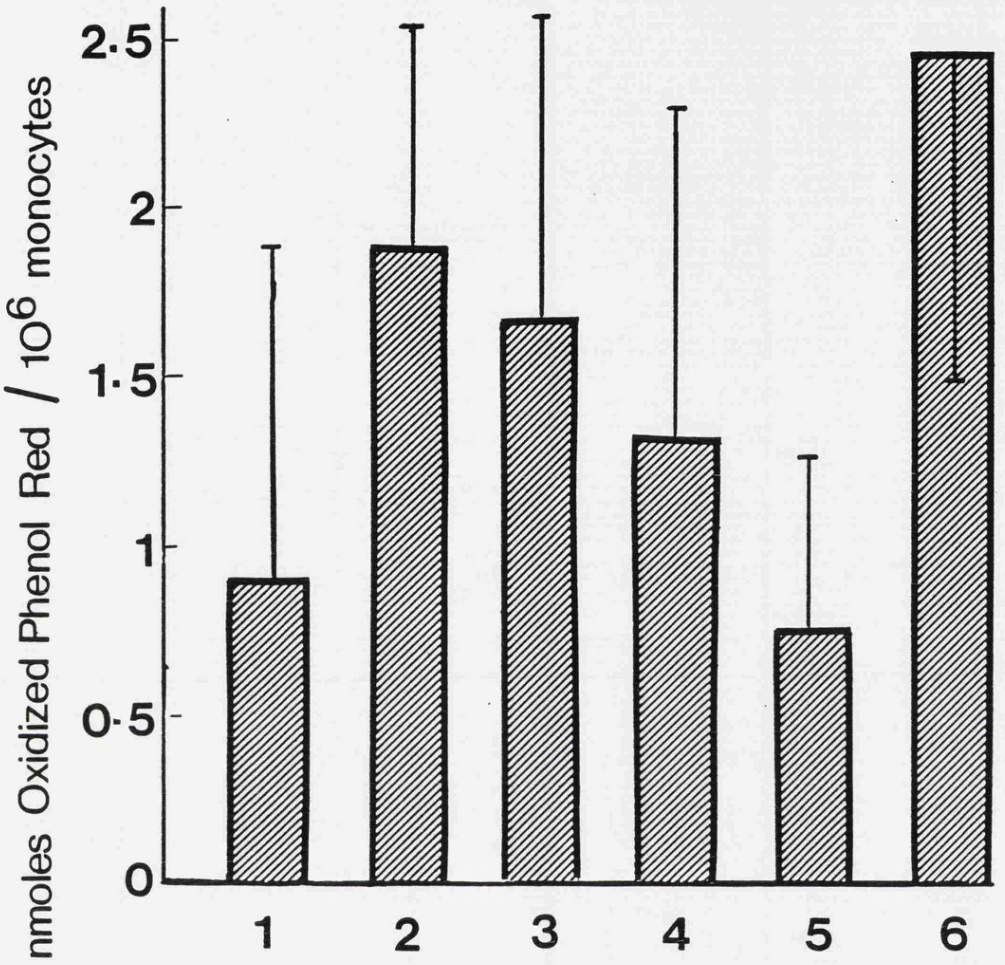


Figure 3.16 H_2O_2 and ROOH release by 1 x 10^6 monocytes over 30 minutes at 37°C stimulated by HAGG, FMLP and opsonized zymosan (corrected)

Histogram	Peroxide	Stimulus
1	H_2O_2	Unstimulated
2	H_2O_2	HAGG 200µg
3	ROOH	HAGG 200µg
4	H_2O_2	FMLP 20µM
5	H_2O_2	Opsonized Zymosan 800µg
6	ROOH	Opsonized Zymosan 800µg

Results are expressed $\bar{X}$ ± SD of 15 experiments.

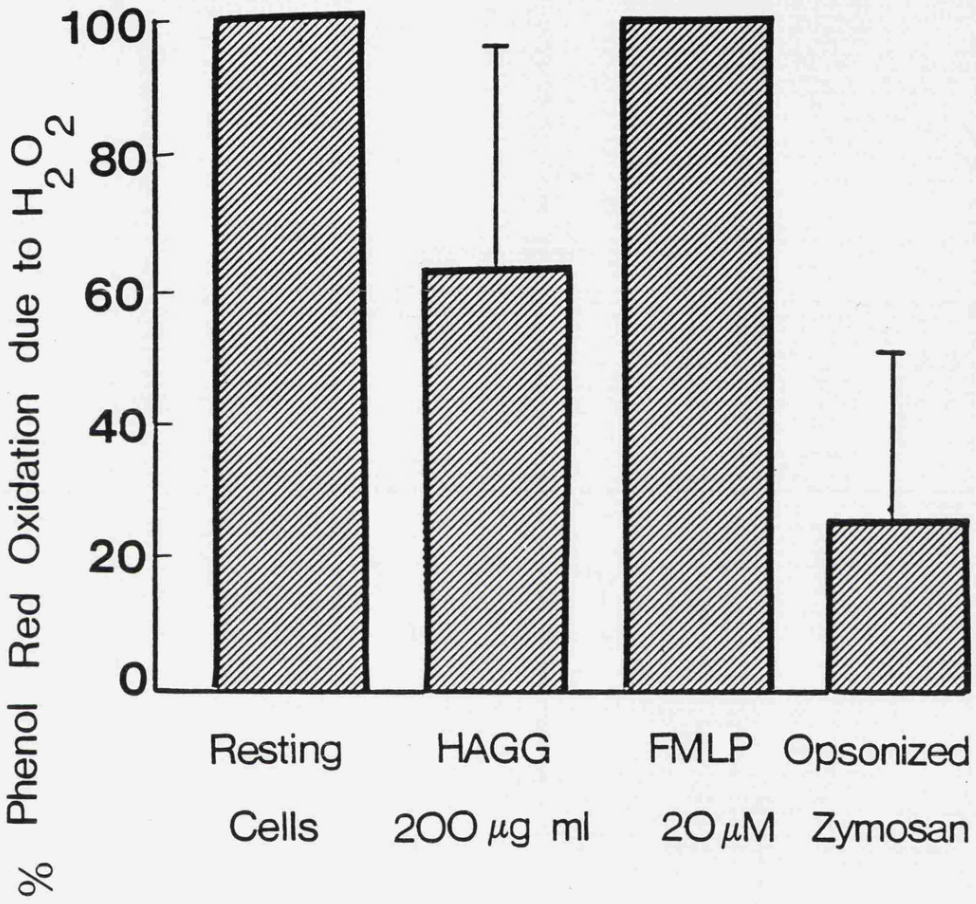


Figure 3.17 % of phenol red oxidation due to H_2O_2 secretion by 1×10^6 monocytes in response to stimuli and by cells alone. Results presented as $\bar{X} \pm SD$ of 15 experiments.

monocytes generate 25% hydrogen peroxide and 2 x 10^6/ml neutrophils generate 11% hydrogen peroxide. As is the case with neutrophils, unstimulated monocytes and monocytes stimulated by FMLP (20μM) produce only hydrogen peroxide and not ROOH.

CHAPTER FOUR

RESULTS B

4. THE EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND OTHER INHIBITORS OF ARACHIDONIC ACID METABOLISM ON NEUTROPHIL OXIDATIVE METABOLISM

The ability of stimulated neutrophils to produce H_2O_2 and ROOH was examined in the presence of various inhibitors of arachidonic acid metabolism using the phenol red assay.

Before the inhibitors were incubated with the cells they were first tested in the assay to determine whether or not they had any direct effect on phenol red oxidation which would produce artefactual inhibitory results. This was achieved by incubating buffered phenol red solution with the inhibitors in the presence of a known amount of H_2O_2 at 37°C for 30 minutes. Once any direct effects had been established, a range of concentrations of each inhibitor were incubated with cells at a concentration of 2×10^6/ml in the presence of HAGG or FMLP and in the presence or absence of catalase at 37°C for 30 minutes. Cell viability was checked at the end of the incubation period by trypan blue exclusion.

4.1.1 Cyclooxygenase Inhibitors

The results for cyclooxygenase inhibitors are shown in Figures 4.1 - 4.5 and are expressed as % inhibition of the phenol red oxidation observed for 2×10^6 stimulated cells alone and in the absence of drug or catalase. The inhibitors used were indomethacin (2.8×10^{-4}M - 2.8×10^{-6}M); diclofenac sodium (6×10^{-4}M - 6×10^{-7}M); fenclofenac (3×10^{-4}M - 3×10^{-6}M) and piroxicam (10^{-4}M - 5×10^{-7}M).

Indomethacin (Figure 4.1) had no direct effect on the phenol red assay and no effect on cell viability at the concentrations tested. This drug showed a small dose-dependent inhibition of phenol red oxidation

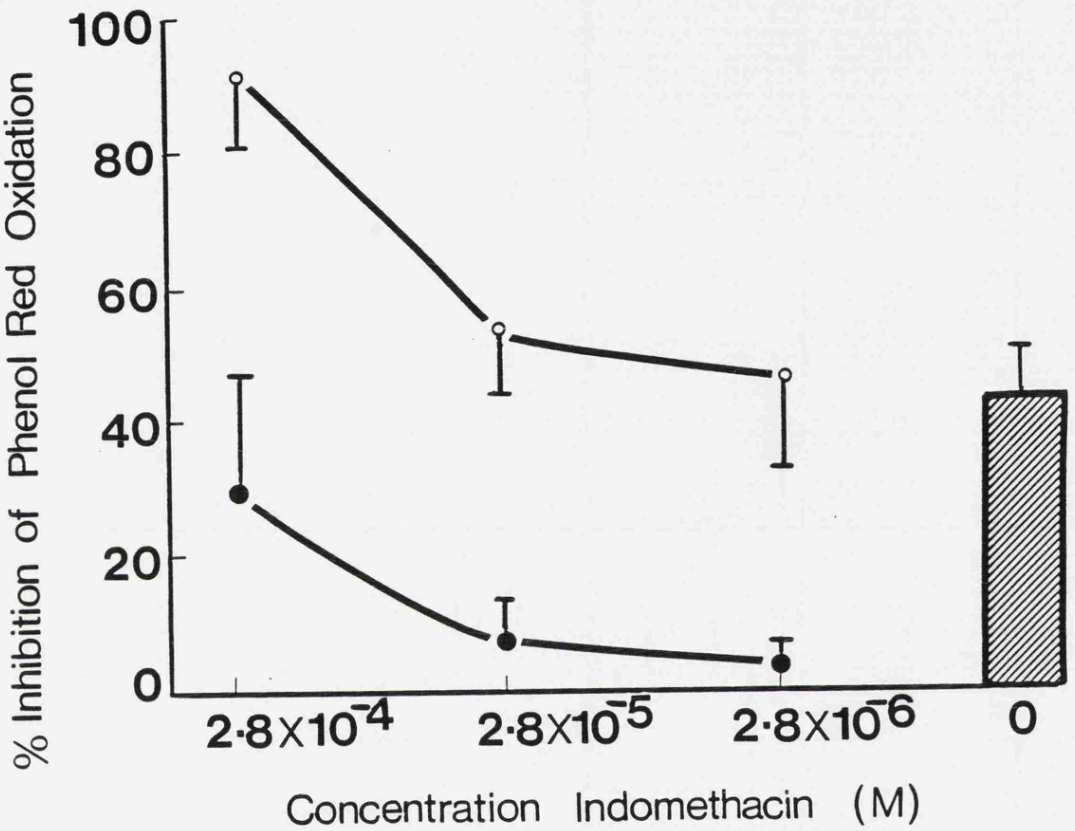


Figure 4.1 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by 200µg HAGG: % inhibition in the presence of indomethacin.

● Stimulated cells + drug

○ Stimulated cells + drug + catalase

Bar Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 3 experiments
Incubation was for 30 minutes at 37°C.

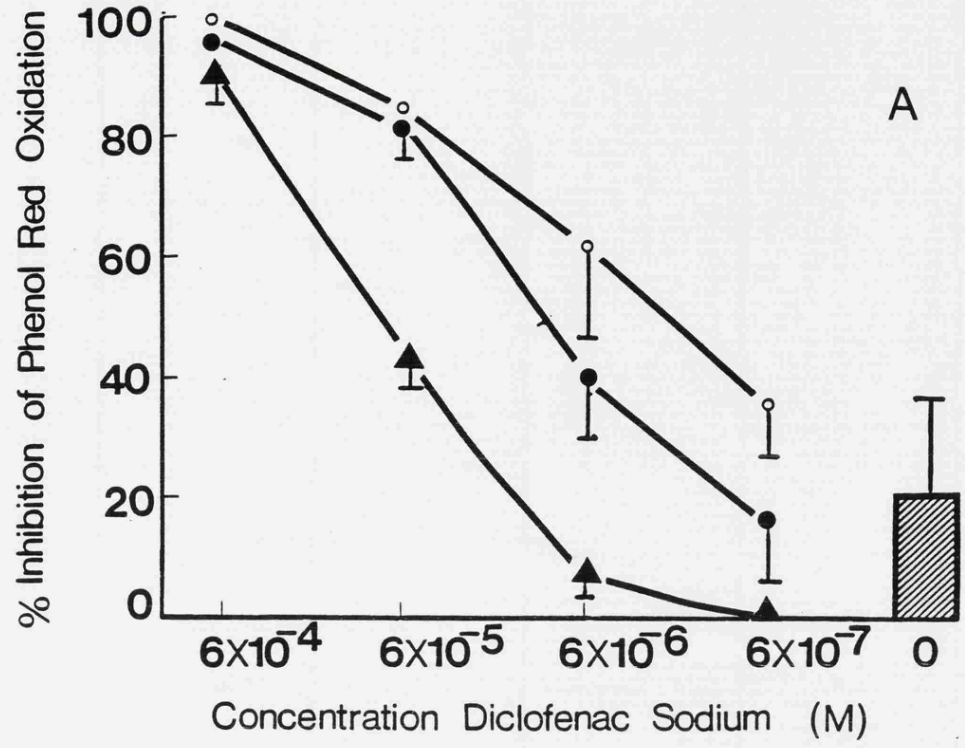
with 30% inhibition achieved at a concentration of 2.8×10^{-4}M, falling to only 2% at 2.8×10^{-6}M.

Diclofenac sodium (Figures 4.2 - 4.3) had no effect on cell viability at the concentrations tested. However it had a direct dose-dependent inhibitory effect on phenol red oxidation as shown, causing 90% inhibition at a concentration of 6×10^{-4}M, falling to 9% at 6×10^{-6}M and 0% at 6×10^{-7}M. In spite of this, the drug still showed a dose-dependent inhibition of phenol red oxidation by neutrophils in response to stimulation by HAGG. Taking into account the direct effect on the assay by the drug this inhibition is approximately 40% at 6×10^{-5}M, 30% at 6×10^{-6}M and 18% at 6 $\times 10^{-7}$M. Thus diclofenac sodium is more potent than indomethacin at inhibiting phenol red oxidation by neutrophils in response to stimulation by HAGG.

However, the results shown for the effect on HAGG-stimulated monocyte phenol red oxidation indicate that the drug had little if any effect on these cells. Catalase inhibits a higher proportion of phenol red oxidation caused by HAGG-stimulated monocytes than by neutrophils stimulated with HAGG i.e. monocytes release a higher proportion (but not absolute amount) of H_2O_2 in response to stimulation than do neutrophils. This confirms results shown in Chapter 3.

Diclofenac sodium also showed a dose-dependent inhibition of phenol red oxidation by neutrophils stimulated by FMLP. This parallels the effect on HAGG-stimulated phenol red oxidation, achieving 35% inhibition at 6×10^{-5}M, 35% at 6×10^{-6}M and 23% at 6×10^{-7}M, taking into account the direct effect on the assay. However, unlike that stimulated by HAGG, this oxidation is all catalase-sensitive i.e. it is all due to H_2O_2 (Figure 4.3).

Fenclofenac (Figure 4.4) had no direct effect on the assay over the chosen concentration range. However, 10% cell death was observed at a



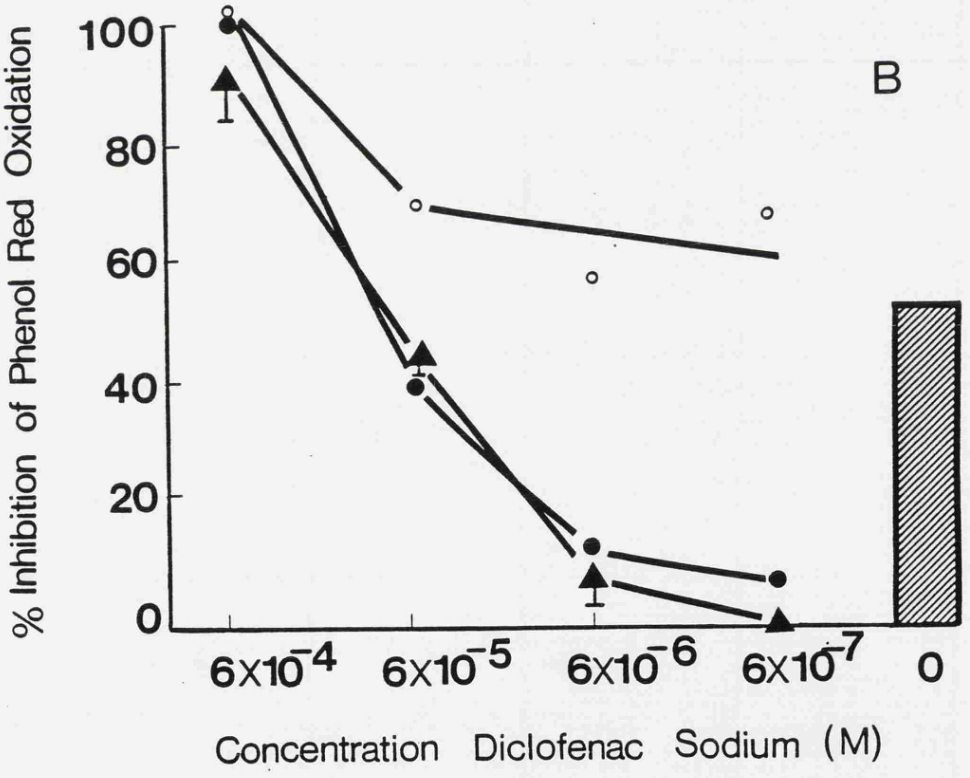


Figure 4.2 Phenol red oxidation by A: 2 x 10^6 neutrophils; B: 1 x 10^6 monocytes stimulated by 200µg HAGG: % inhibition in the presence of diclofenac sodium.

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ Direct effect of drug on assay
Bar Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 3 experiments
Incubation was for 30 minutes at 37°C.

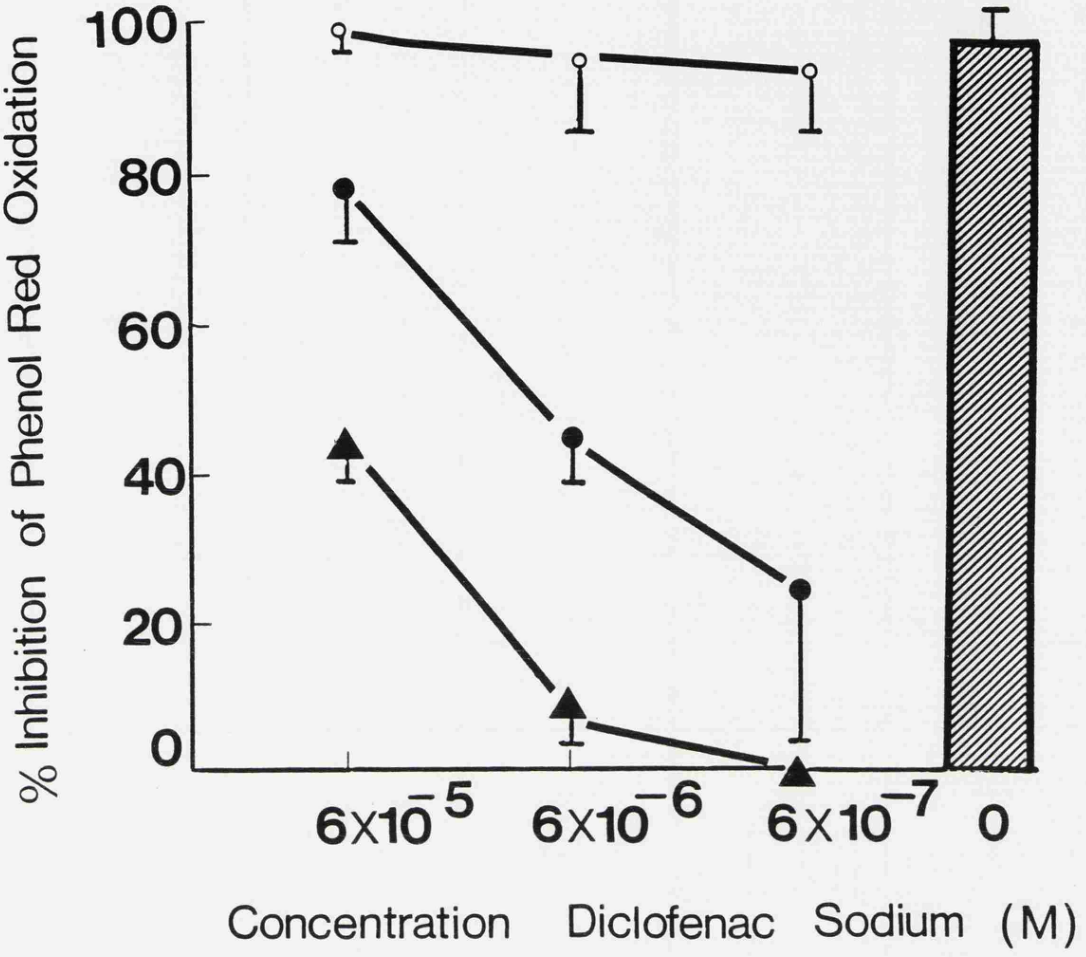


Figure 4.3 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by FMLP (20μM): % inhibition in the presence of diclofenac sodium.

● Stimulated cells + drug

○ Stimulated cells + drug + catalase

▲ Direct effect of drug on assay

Bar Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 3 experiments
Incubation was for 30 minutes at 37°C.

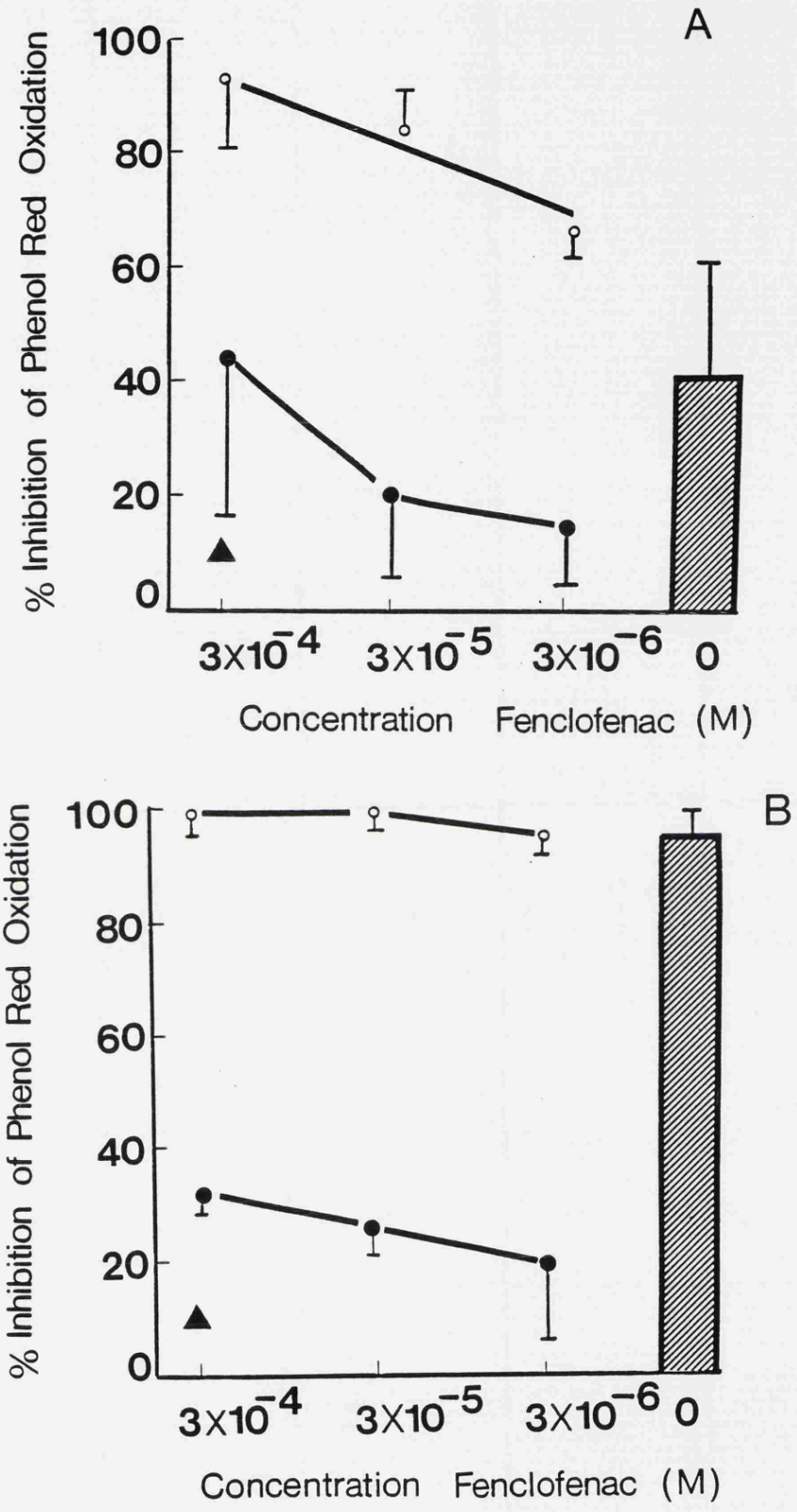


Figure 4.4 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by A: 200µg HAGG; B: FMLP (20µM): % inhibition in the presence of fenclofenac.

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ % cell death
Bar Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 4 experiments.
Incubation was for 30 minutes at 37°C.

concentration of 3×10^{-4}M. Fenclofenac shows a dose-dependent inhibition of phenol red oxidation by neutrophils stimulated by HAGG, achieving 35% inhibition at a concentration of 3×10^{-4}M (taking into account cell death), falling to 14% at 3×10^{-6}M. This drug also inhibits FMLP-stimulated phenol red oxidation by neutrophils, achieving 25% inhibition at 3×10^{-4}M and 20% at 3×10^{-6}M. Thus, whilst fenclofenac is more potent than indomethacin, it is less potent than diclofenac sodium at inhibiting phenol red oxidation by stimulated neutrophils.

Piroxicam (Figure 4.5) had no direct effect on the assay over the concentration range tested, but at 10^{-4}M it did cause 5% cell death. Inhibition of phenol red oxidation by neutrophils stimulated by HAGG is slight, with 29% inhibition achieved at 10^{-4}M. Moreover, inhibition by this drug of phenol red oxidation by FMLP-stimulated neutrophils, is also very slight and is not dose-related, with 24% inhibition achieved at 10^{-4}M.

A new cyclooxygenase inhibitor, pirprofen, a propionic acid derivative, was also tested in the assay system, but proved to interfere directly with the phenol red assay and so was not used with cells. As Table 4.1 shows, pirprofen itself prevents phenol red oxidation by H_2O_2, giving an apparent inhibition of phenol red oxidation of 97% at 10^{-3}M and becoming negligible at 5×10^{-6}M.

4.1.2 Lipoxygenase Inhibitors

Nordihydroguaiaretic acid (NDGA) (Figure 4.6) has marked anti-oxidant properties at high concentrations and thus proved to have a direct effect on phenol red oxidation, achieving 30% inhibition at 5×10^{-6}M falling to 0% at 5×10^{-7}M. It also caused 10% cell death at 5×10^{-6}M.

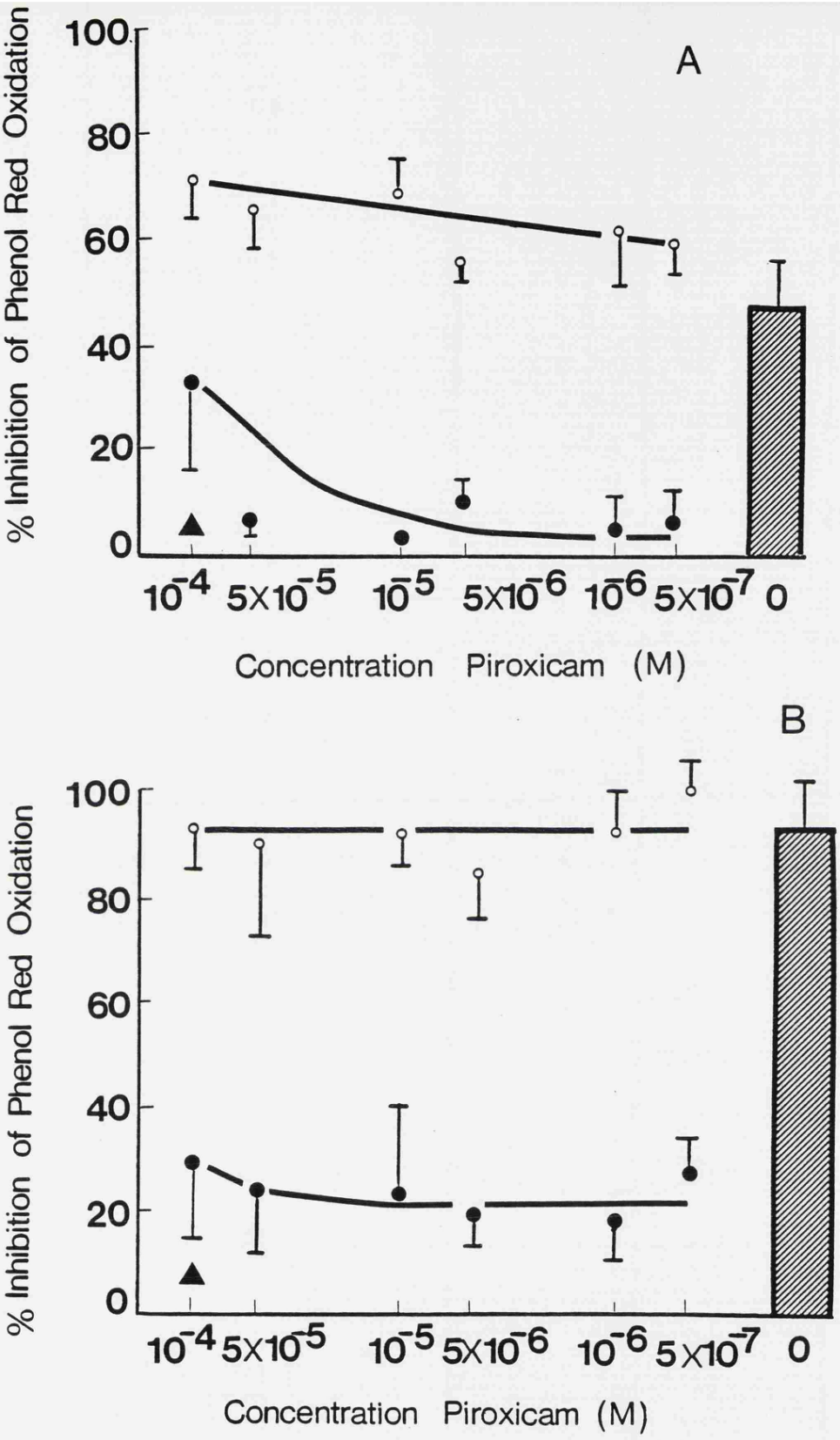


Figure 4.5 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by A: 200µg HAGG; B: FMLP (20µM): % inhibition in the presence of piroxicam.

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ % cell death
Bar Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 3 experiments.
Incubation was for 30 minutes at 37°C.

Concentration pirprofen (M)	Absorbance at 610nm in the presence of 15nmoles H_2O_2	Apparent % inhibition of phenol red oxidation
0	0.444	0
10^{-3}	0.014	97
5×10^{-4}	0.010	98
10^{-4}	0.150	76
5×10^{-5}	0.257	42
10^{-5}	0.307	31
5×10^{-6}	0.450	0
10^{-6}	0.465	0

Table 4.1 The direct effect of various concentrations of pirprofen on the phenol red assay. Each incubation was set up in duplicate and was carried out at room temperature for 30 minutes.

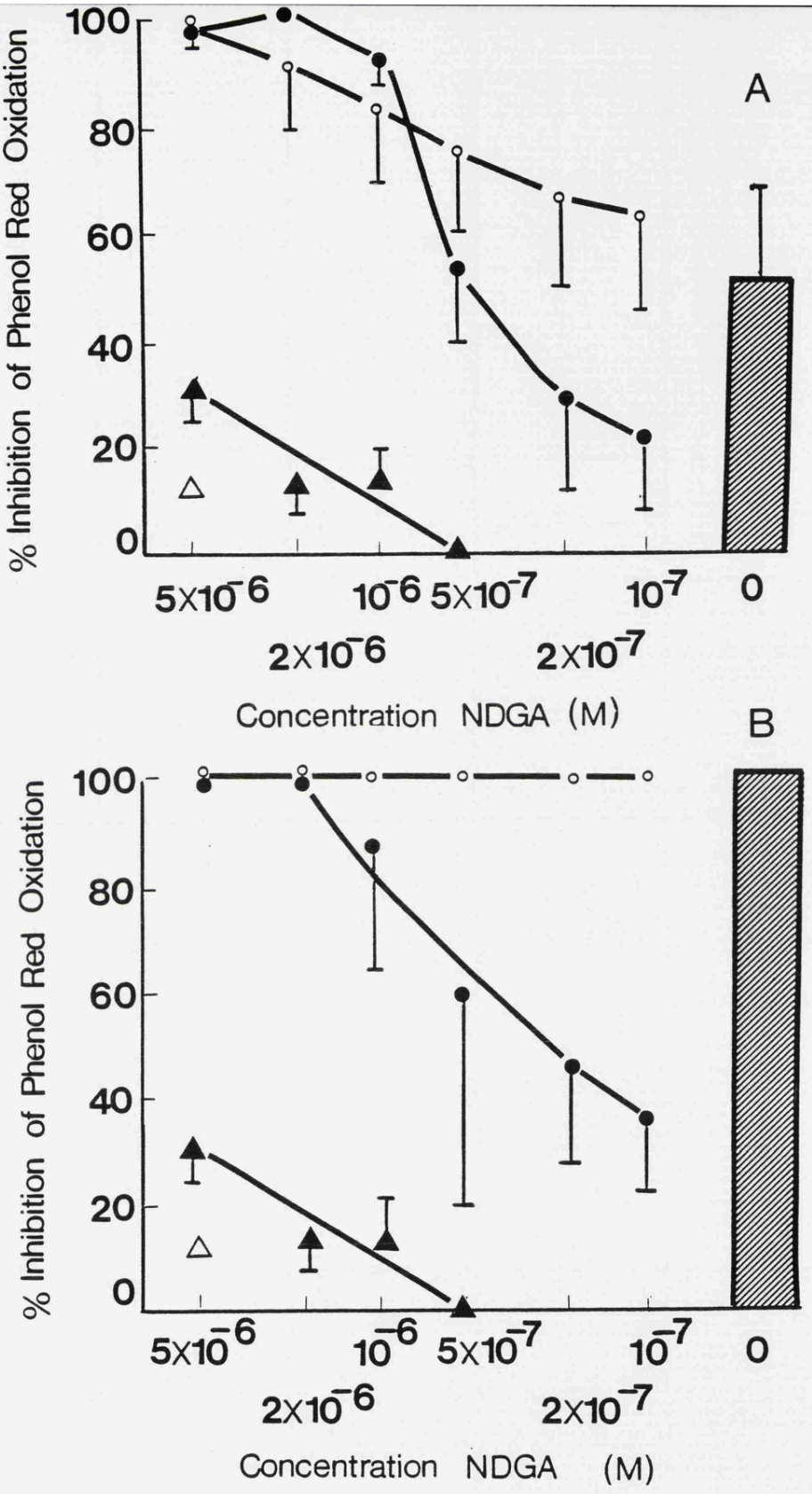


Figure 4.6 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by A: 200µg HAGG; B: FMLP (20µM): % inhibition in the presence of NDGA.

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ Direct effect of drug on assay
△ % cell death
Bar Stimulated cells + catalase

Results expressed as $\bar{X} \pm SD$ of 4 experiments.
Incubation was for 30 minutes at 37°C.

However, NDGA showed a dose-dependent inhibition of phenol red oxidation by neutrophils stimulated by HAGG, with an inhibitory effect of 85% at 2 x 10^{-6}M and 23% at 10^{-7}M, taking into account the direct effect on the assay. Moreover, it also showed an inhibitory effect on FMLP-stimulated neutrophil-mediated phenol red oxidation achieving again 85% inhibition at 2 x 10^{-6}M and 36% at 10^{-7}M. Thus, NDGA is much more potent than any of the cyclooxygenase inhibitors at inhibiting phenol red oxidation by stimulated neutrophils.

4.1.3 Cyclooxygenase/Lipoxygenase Inhibitors

Benoxaprofen (Figure 4.7) proved to have no direct effect on phenol red oxidation over the concentration range tested, but did cause 10% cell death at 10^{-3}M. Whilst it did show a dose-dependent inhibition of phenol red oxidation by HAGG-stimulated neutrophils, its effect was not very potent, with an inhibition of 67% at 10^{-3}M which fell rapidly to 10% at 10^{-5}M.

BW 755C (Figure 4.8) had a marked direct effect on phenol red oxidation, with 100% inhibition at 10^{-4}M. However, this fell to 30% at 5 x 10^{-6}M and 3% at 10^{-6}M, and at these concentrations it caused an inhibition of phenol red oxidation by HAGG-stimulated neutrophils of 34% at 5 x 10^{-6}M and 20% at 10^{-6}M. This was above that caused by a direct effect on the assay. It did not have any effect on cell viability at the concentrations tested.

4.1.4 Phospholipase A_2 Inhibitors

Quinacrine (Figure 4.9) could only be used over a narrow range of concentrations (10^{-4}M - 5 x 10^{-6}M) as it proved to directly affect phenol red oxidation at higher concentrations. It caused 30% cell death at a concentration of 10^{-4}M and 10% cell death at 5 x 10^{-5}M.

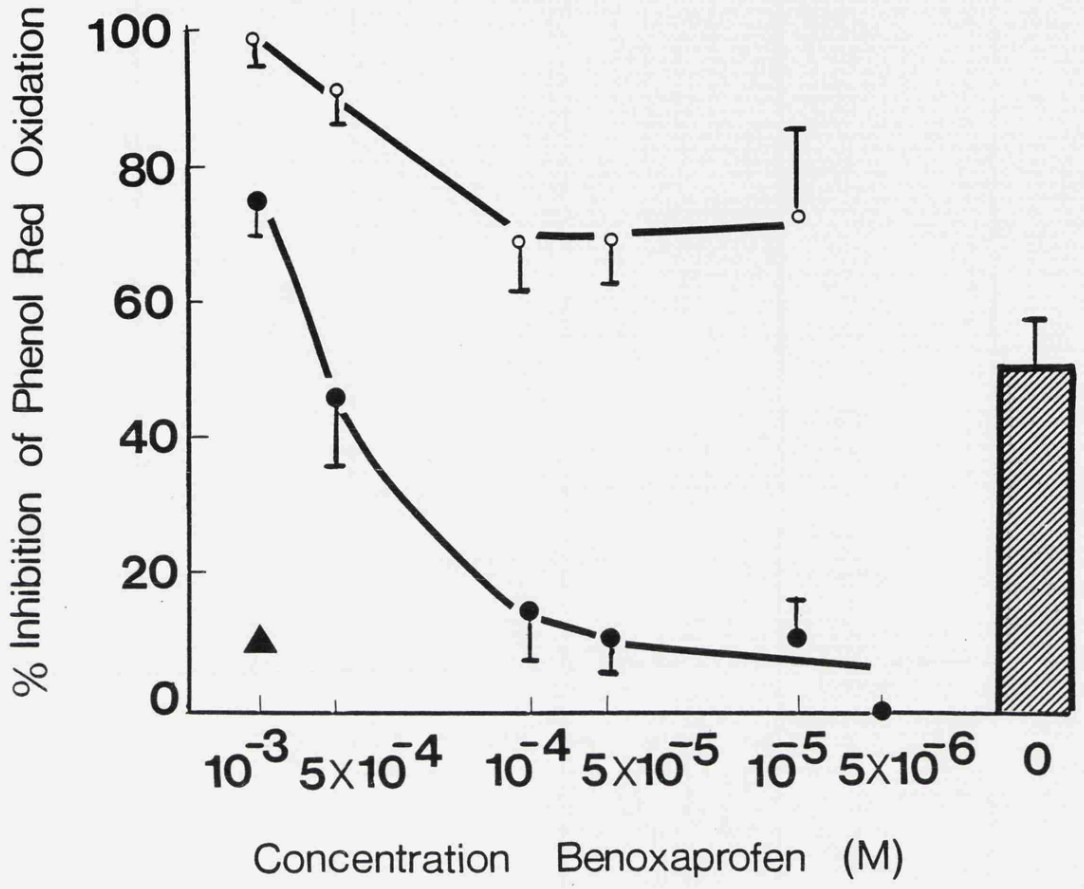


Figure 4.7 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by 200µg HAGG: % inhibition in the presence of benoxaprofen.

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ % cell death
Bar Stimulated cells + catalase

Results expressed as $\overline{X}$ ± SD of 3 experiments.
Incubation was for 30 minutes at 37°C.

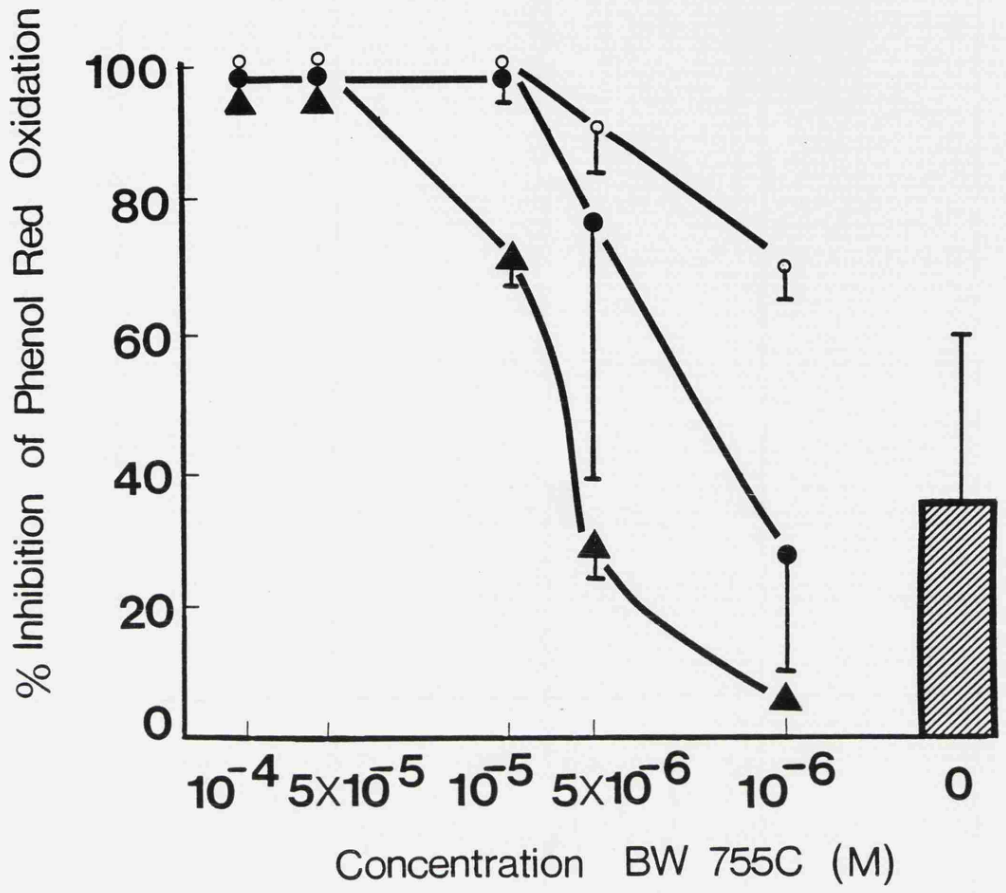


Figure 4.8 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by 200µg HAGG: % inhibition in the presence of BW 755C.

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ Direct effect of drug on assay
Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 3 experiments.
Incubation was for 30 minutes at 37°C.

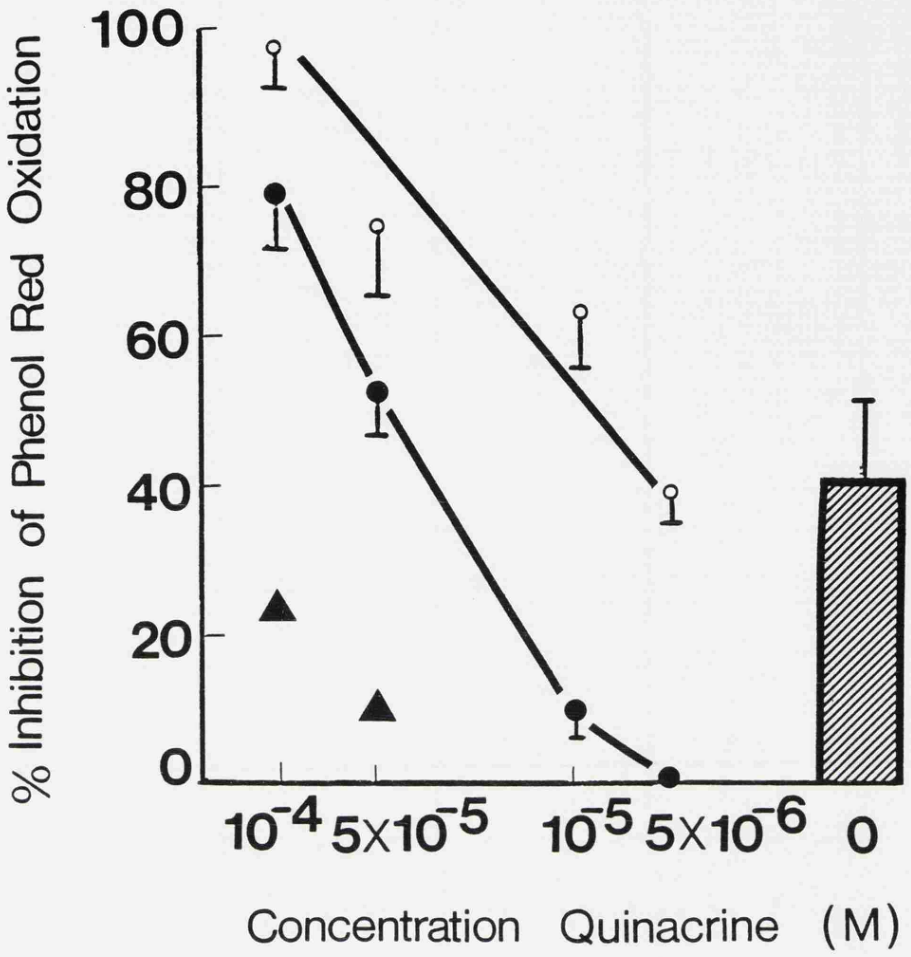


Figure 4.9 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by 200µg HAGG: % inhibition in the presence of quinacrine

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ % cell death
Bar Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 3 experiments.
Incubation was for 30 minutes at 37°C.

Correcting for this, the drug inhibited phenol red oxidation by HAGG-stimulated neutrophils by 50% at 10^{-4}M, falling to 10% at 10^{-5}M and with no effect at 5 x 10^{-6}M. It thus proved to be not very potent.

p-Bromophenacyl bromide (pBPB) had no direct effect on the assay and only caused 5% cell death at a concentration of 5 x 10^{-5}M. It showed a potent dose-dependent inhibition of phenol red oxidation by neutrophils stimulated by HAGG, with 100% inhibition at 10^{-5}M and 22% inhibition at 5 x 10^{-7}M. Similarly it had a marked inhibitory effect on phenol red oxidation by FMLP-stimulated neutrophils, achieving 100% inhibition at 10^{-5}M and 46% inhibition at 5 x 10^{-7}M, (Figure 4.10).

4.1.5 Sulphydryl Group Blocker

The effects of the SH group blocker p-hydroxymercuriphenylsulphonic acid (pHMPSA) on the production of H_2O_2 and ROOH by 1 x 10^6 neutrophils and 1 x 10^6 monocytes were also investigated. After pretreatment of cells with 50μM pHMPSA for 1 hour at 37°C, production of both H_2O_2 and ROOH by neutrophils and monocytes in response to stimulation by HAGG, FMLP or opsonized zymosan was completely inhibited (Table 4.2). There was no effect on cell viability.

In addition, a dose-response experiment was performed using pHMPSA over a concentration range of 1μM - 50μM. After pretreatment, cells were stimulated with HAGG in parallel with untreated cells (Figure 4.11). Unfortunately this experiment was not performed in the presence of catalase, so the effects on the production of H_2O_2 and ROOH cannot be evaluated separately. However, a dose-dependent inhibition of phenol red oxidation could be observed, with total inhibition at 25μM and an ID_{50} of 5μM.

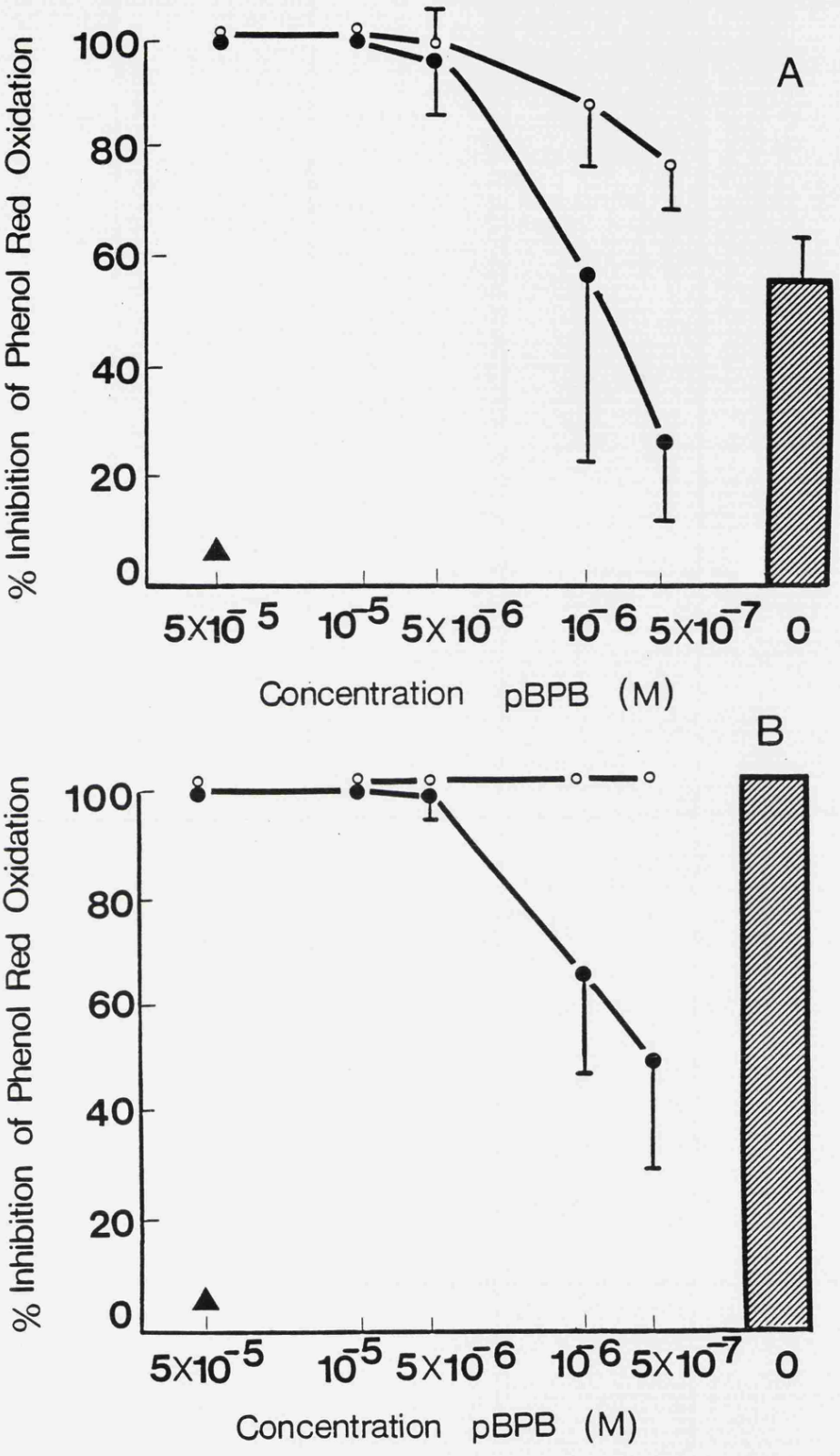


Figure 4.10 Phenol red oxidation by 2 x 10^6 neutrophils stimulated by A: 200µg HAGG; B: FMLP (20µM): % inhibition in the presence of pBPB.

● Stimulated cells + drug
○ Stimulated cells + drug + catalase
▲ % cell death
Bar Stimulated cells + catalase

Results expressed as $\bar{X}$ ± SD of 3 experiments.
Incubation was for 30 minutes at 37°C.

Cell ($1x10^6$/ml)	Stimulus	nmoles ROOH -pHMPSA	nmoles ROOH +pHMPSA	nmoles H_2O_2 -pHMPSA	nmoles H_2O_2 +pHMPSA
Neutrophil	HAGG 200µg	11	0	6	0
	FMLP 20µM	0	0	11.5	0
	Opsonized Zymosan 800µg	5	0	0.5	0
Monocyte	HAGG 200µg	0.5	0	2	0
	Opsonized Zymosan 800µg	0.5	0	1	0

Table 4.2: The effect of pre-treatment of 1 x 10^6 neutrophils and monocytes by 50µM pHMPSA for 1 hour at 37°C on subsequent ROOH and H_2O_2 production in response to given stimuli.

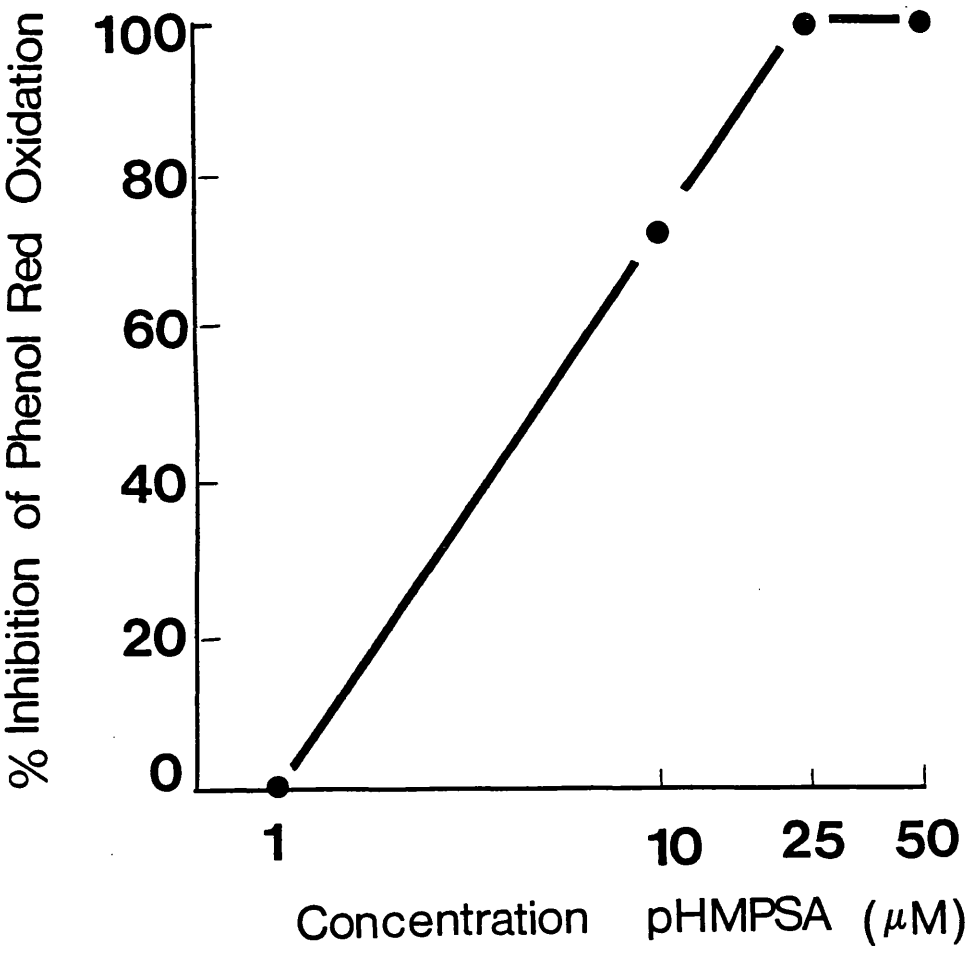


Figure 4.11 Oxidation of phenol red by 1 x 10^6 neutrophils stimulated by 200μg HAGG: % inhibition by pre-treatment of neutrophils with pHMPSA for 1 hour at 37°C.

4.1.6 Effect of Inhibitors on Each Peroxide Product

In order to determine the effect the inhibitors have on each component of the phenol red oxidation, the following equations were devised:

1. % Inhibition of oxidation caused by ROOH due to the presence of the inhibitor.

Working on the assumption that the presence of catalase prevents any of the oxidation of phenol red being due to H_2O_2 and that ROOH is catalase insensitive, then subtracting the catalase sensitive portion of the phenol red oxidation from % inhibition for inhibitor + catalase gives the effect of the inhibitor on ROOH production. If this is then divided by % catalase insensitive, this gives the % inhibition of oxidation caused by ROOH in the presence of the inhibitor.

i.e.,

$$\frac{(\%\ \text{Inhibition for inhibitor + catalase} - \%\ \text{catalase sensitive})}{(100\% - \%\ \text{catalase sensitive})} \times 100\% \quad \text{(A)}$$

2. % Inhibition of oxidation caused by H_2O_2 due to the presence of the inhibitor.

To determine the effect of the inhibitor on H_2O_2 production, if the value obtained in (A) above i.e. the effect of the inhibitor on ROOH is subtracted from the value obtained for the % inhibition by inhibitor alone i.e. the effect of the inhibitor on both peroxides, then this gives the effect the inhibitor must be having on H_2O_2 production. This can then be expressed as a % of the catalase sensitive portion of oxidation to give % inhibition of oxidation caused by H_2O_2 due to the presence of the inhibitor.

i.e.,

$$\frac{(\%\ \text{Inhibition by inhibitor alone} - (\text{A}))}{\%\ \text{catalase sensitive}} \times 100\%$$

Using figures obtained for inhibition by 10^{-6}M pBPB of HAGG-stimulated phenol red oxidation as an example (from Figure 4.10)

% Inhibition by pBPB alone = 58%

% Inhibition by catalase alone = 55%

% Inhibition by pBPB + catalase = 84%

Applying equation 1:

$$\frac{84 - 55}{100 - 55} \times 100 = \frac{29}{45} \times 100 = 64\%$$

Applying equation 2:

$$\frac{58 - (84 - 55)}{55} \times 100 = \frac{29}{55} \times 100 = 53\%$$

Thus applying these equations to the results obtained with all the inhibitors gives the results shown in Figures 4.12 - 4.13.
The ED_{50} values can be calculated and are shown in Table 4.3. pBPB and NDGA prove to be the most potent inhibitors of ROOH production (ED_{50} = 5 x 10^{-7}M) and H_2O_2 production (ED_{50} = 10^{-6}M and 4 x 10^{-7}M respectively). Indomethacin and piroxicam are the weakest inhibitors of ROOH production (ED_{50} = 10^{-4}M and >10^{-4}M respectively). Fenclofenac, benoxaprofen, indomethacin and piroxicam all seem to have a "negative inhibitory" effect on H_2O_2 production (see section 4.3).
A summary of the effects the different classes of inhibitors have on neutrophil production of ROOH and H_2O_2 in response to stimulation with HAGG or FMLP is shown in Table 4.4. There is an anomaly in the effect cyclooxygenase inhibitors have on H_2O_2 production depending upon whether the stimulus is HAGG or FMLP.

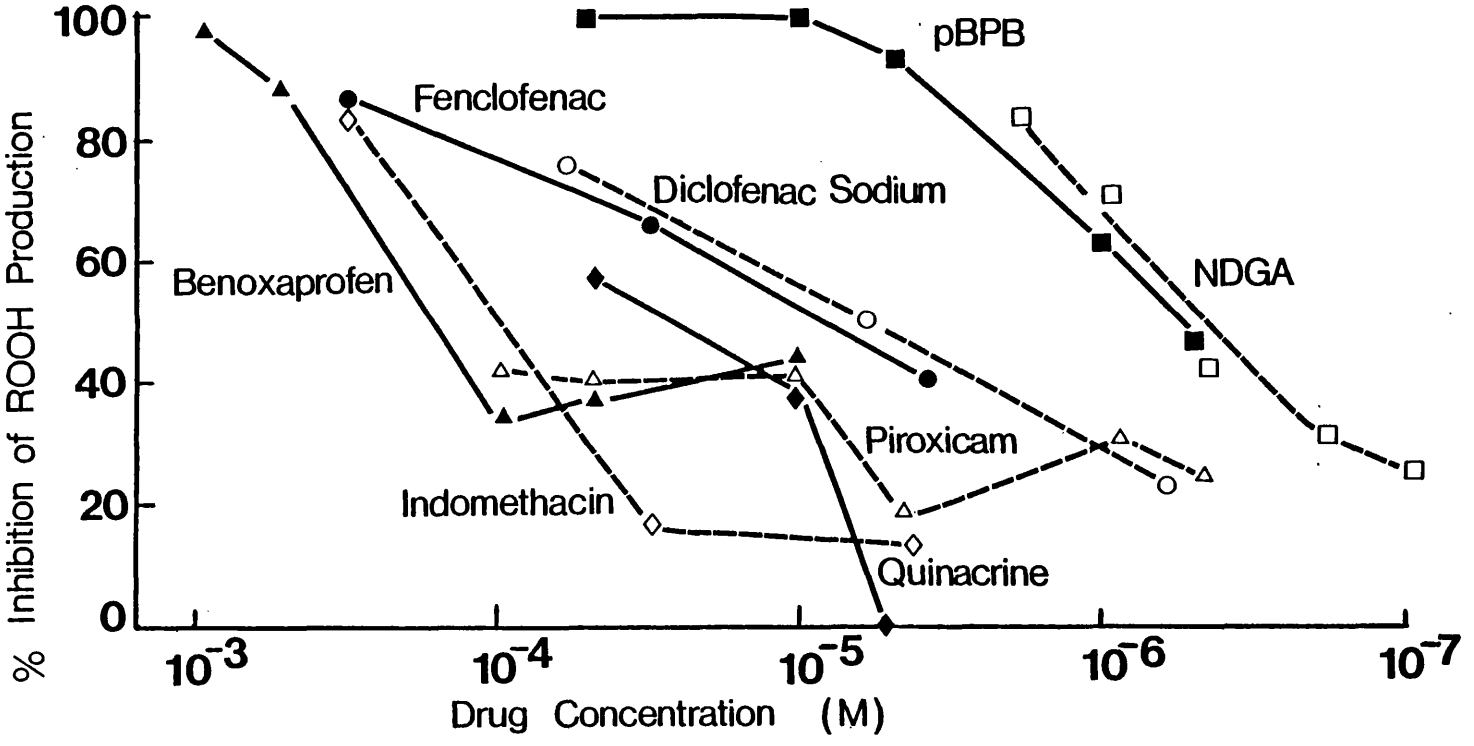


Figure 4.12 ROOH production by 2 x 10^6 neutrophils stimulated by 200µg HAGG: % inhibition by various inhibitors.

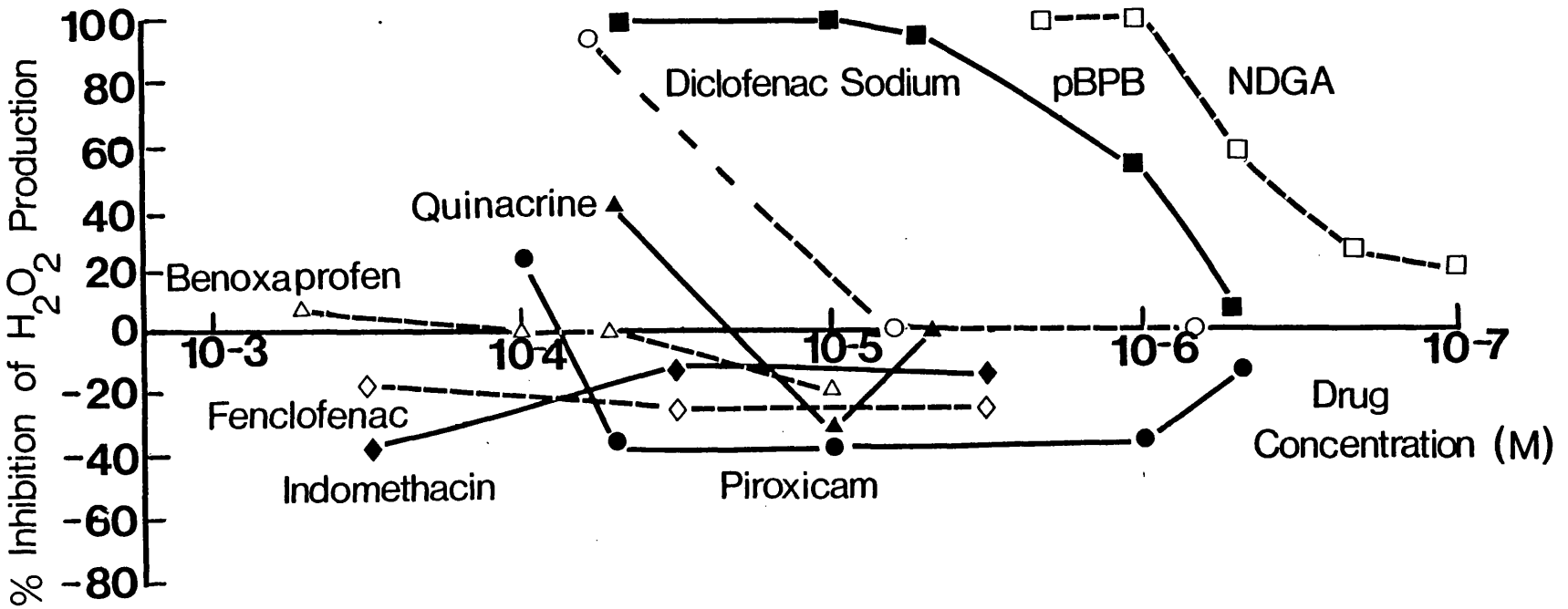


Figure 4.13 H_2O_2 production by 2 x 10^6 neutrophils stimulated by 200µg HAGG: % inhibition by various inhibitors.

Inhibitor	ED_{50} for Inhibition of ROOH (M)	ED_{50} for Inhibition of H_2O_2 (M)
pBPB	5×10^{-7}	10^{-6}
NDGA	5×10^{-7}	4×10^{-7}
Diclofenac sodium	6×10^{-6}	2×10^{-5} *
Fenclofenac	6×10^{-6}	"negative inhibition"
Quinacrine	3×10^{-5}	inactive
Benoxaprofen	2×10^{-4}	"negative inhibition"
Indomethacin	10^{-4}	"negative inhibition"
Piroxicam	$>10^{-4}$	"negative inhibition"

*This is difficult to assess accurately because of its direct effect on the assay (44% inhibition at 6×10^{-5}M and 9% at 6×10^{-6}M).

Table 4.3: ED_{50} values for inhibition of both peroxides produced by HAGG-stimulated neutrophils by various inhibitors.

Drug class	Effects on 2×10^6 neutrophils		
	Effect on ROOH production	Effect on H_2O_2 production	
		HAGG	FMLP
Cyclooxygenase Inhibitors	Inhibitory	"-ve Inhibitory" or none	Inhibitory
Lipoxygenase Inhibitors	Inhibitory	Inhibitory	Inhibitory
Cyclooxygenase/ Lipoxygenase Inhibitors	Inhibitory	"-ve Inhibitory"	----
Phospholipase Inhibitors	Inhibitory	Inhibitory	Inhibitory

Table 4.4: Summary of effects classes of drugs have on neutrophil production of ROOH and H_2O_2.

4.2 Structure - Activity Relationships

From these results it would appear that cyclooxygenase inhibitors are having effects on neutrophils unrelated to their ability to inhibit cyclooxygenase. It was decided to investigate whether or not these effects on neutrophil activity were due to any aspect of their structure and thus some analogues of diclofenac sodium and fenclofenac were kindly supplied by the manufacturers, Ciba Geigy and Reckitt and Coleman. Their effects on neutrophils were tested using the phenol red assay and compared with the marketed compounds.

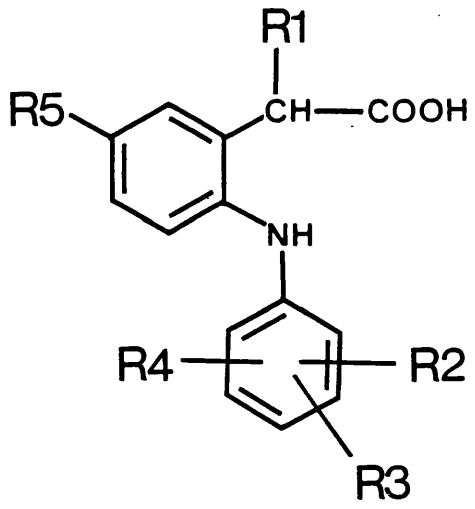
4.2.1 Diclofenac Analogues

The structures of the analogues are shown in Figure 4.14. The concentration chosen was 6×10^{-6}M. This concentration of diclofenac apparently inhibits total phenol red oxidation by 40% (Figure 4.2) but only affects the assay directly by 9% (Figure 4.2).

The ability for the analogues to directly inhibit phenol red oxidation was investigated using the method already described. The results are shown in Table 4.5 and compared with the effect of diclofenac sodium itself on the assay at the same concentration. These results show that all the analogues have a similar effect on the assay to diclofenac sodium except analogue 2 which has a marked direct inhibitory effect on the oxidation of phenol red by H_2O_2.

The analogues were then incubated at 6×10^{-6}M with 2×10^{6} neutrophils stimulated with 200µg HAGG or FMLP 20µM for 30 minutes at 37°C in the presence or absence of catalase. The results for % inhibition of total phenol red oxidation and % inhibition of ROOH production and H_2O_2 production are shown in Table 4.5.

Structure



Analogue	R1	R2	R3	R4	R5
Diclofenac	H	2-Cl	H	6-Cl	H
2	H	2-CH_3	H	6-CH_3	H
6	H	2-Cl	4-OCH_3	6-Cl	H
7	H	2-Cl	H	6-Cl	Cl
15	H	3-CF_3	H	5-CF_3	H
18	CH_3	2-Cl	H	6-Cl	H
19	H	2-Cl	H	H	H
20	H	2-Cl	4-Cl	H	H
21	H	2-Cl	3-Cl	H	H

Figure 4.14 Structures of diclofenac sodium and its analogues.

Analogue Number	Direct % inhibition of phenol red assay $\bar{X}$ ± SD n=3	% Inhibition of phenol red oxidation Stimulus: HAGG n=3		% Inhibition of phenol red oxidation Stimulus: FMLP n=4		% Inhibition of ROOH Stimulus: HAGG	% Inhibition of H_2O_2 Stimulus: HAGG	% Inhibition of H_2O_2 Stimulus: FMLP
		-catalase	+catalase	-catalase	+catalase			
None	----	----	51 ± 12	----	98 ± 4	----	----	----
2	53 ± 4	69 ± 10	86 ± 9	57 ± 7	96 ± 4	71	67	57
6	16 ± 5	44 ± 4	81 ± 9	41 ± 8	95 ± 5	61	27	41
7	4 ± 1	35 ± 5	80 ± 10	31 ± 13	96 ± 4	59	12	31
15	0 ± 0	0	67 ± 14	16 ± 13	94 ± 7	33	-31	16
18	8 ± 2	43 ± 2	80 ± 7	49 ± 10	95 ± 5	59	27	49
19	12 ± 2	14 ± 3	80 ± 4	28 ± 11	95 ± 6	59	-29	28
20	13 ± 0	33 ± 2	77 ± 9	39 ± 11	95 ± 6	52	9	39
21	6 ± 3	4 ± 7	78 ± 15	24 ± 13	96 ± 4	55	-45	24
Diclofenac	9 ± 4	40 ± 11	61 ± 15	37 ± 17	98 ± 4	51	0	37

Table 4.5 Effect of 6×10^{-6} M diclofenac sodium and its analogues on the oxidation of phenol red by 2×10^{6} neutrophils stimulated with HAGG or FMLP.

These results show that analogues 15, 19 and 21 are much less potent than diclofenac at overall inhibition of phenol red oxidation and they are also the only analogues to have a 'negative inhibitory' effect on H_2O_2 production. Analogue 15 is the only analogue to show a lower potency at inhibiting ROOH production. Analogues 6, 7, 18 and 20 are approximately equipotent with diclofenac. The true effect of analogue 2 is difficult to assess because of its marked direct effect on the assay.

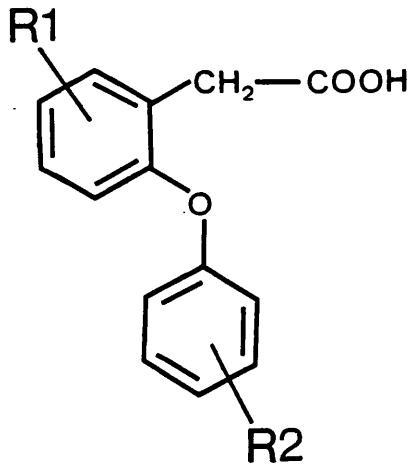
4.2.2 Fenclofenac Analogues

The structures of the analogues are shown in Figure 4.15. The concentration chosen was 1.6×10^{-4}M. As with the diclofenac analogues, the direct effect on the assay was investigated but, in common with fenclofenac, none of the analogues had any direct effect on the oxidation of phenol red by H_2O_2 in repeated experiments.

The analogues were then incubated at 1.6×10^{-4}M with 2×10^6 neutrophils stimulated with 200µg HAGG or FMLP 20µM for 30 minutes at 37°C in the presence or absence of catalase. The results for % inhibition of total phenol red oxidation and % inhibition of ROOH production and H_2O_2 production are shown in Table 4.6.

Analogues 3 and 4 are more potent than fenclofenac at inhibiting overall phenol red oxidation as well as ROOH production and H_2O_2 production due to both stimuli. Analogue 3 is particularly potent at inhibiting H_2O_2 production from FMLP-stimulated neutrophils, achieving 98% inhibition. The other analogues are all equipotent, except that 1, 2 and 8 are more potent at inhibiting H_2O_2 production due to stimulation by HAGG (but not FMLP) and 2 and 6 are less potent at inhibiting ROOH production.

Structure



Analogue	R1	R2
Fenclofenac	H	2,4-Cl_2
1	H	2,4-Cl_2, 3,5-Me_2
2	H	2,4,6-Cl_3
3	5-Me	2Cl, 4-S-Bu
4	H	2,3-Cl_2
5	H	2,4-Me_2
6	H	2-Cl
7	5-Me	2,4-Cl_2
8	5-Cl	2,4-Cl_2

Figure 4.15 Structures of fenclofenac and its analogues.

Analogue Number	% Inhibition of phenol red oxidation Stimulus: HAGG n=4		% Inhibition of phenol red oxidation Stimulus: FMLP n=3		% Inhibition of ROOH Stimulus:	% Inhibition of H_2O_2 Stimulus:	% Inhibition of H_2O_2 Stimulus:
	-catalase	+catalase	-catalase	+catalase	HAGG	HAGG	FMLP
None	----	53 ± 15	----	100	----	----	----
1	34 ± 12	75 ± 4	19 ± 23	100	47	23	19
2	24 ± 11	68 ± 5	26 ± 17	100	32	17	26
3	41 ± 21	82 ± 9	98 ± 4	100	62	23	98
4	37 ± 5	84 ± 4	76 ± 20	100	66	11	76
5	22 ± 6	74 ± 7	30 ± 4	100	45	2	30
6	18 ± 5	72 ± 8	33 ± 1	100	40	-2	33
7	24 ± 11	78 ± 6	31 ± 2	100	53	-2	31
8	32 ± 8	74 ± 5	35 ± 3	100	45	21	35
Fenclofenac	22 ± 9	79 ± 6	27 ± 5	100	55	-8	27

Table 4.6: Effect of 1.6×10^{-4}M fenclofenac and its analogues on the oxidation of phenol red by 2×10^{6} neutrophils stimulated with HAGG or FMLP.

4.3 The Effect of Drugs on Resting Cells

The "negative inhibitory" effects obtained with some inhibitors on H_2O_2 production was puzzling. None of the results with inhibitors had been corrected for resting cell production of H_2O_2 because, although in general this level was very small in comparison with the stimulated levels, it was considered invalid to correct for resting cell levels when it was not known what effect the inhibitors would have on resting cells. However, the "negative inhibitory" results obtained suggested that maybe the inhibitors were actually stimulating resting cells to produce H_2O_2, but not ROOH as there were no "negative inhibitory" results obtained for ROOH production.

To investigate this possibility, 2×10^6 neutrophils were incubated in the presence and absence of a range of concentrations of inhibitors but in the absence of other stimuli for 30 minutes at 37°C in buffered phenol red solution. As the absolute amounts of H_2O_2 generated were small, any % change in absorbance due to the inhibitors was noted. The results are shown in Figure 4.16. Benoxaprofen proved very potent at stimulating increased phenol red oxidation with indomethacin and fenclofenac also having this effect. Piroxicam showed some slight stimulatory activity. However, diclofenac, pBPB and NDGA all inhibited phenol red oxidation.

Comparing this figure with Figure 4.13, the results mirror very accurately what could be predicted, although benoxaprofen is more stimulatory than would be expected from Figure 4.13, increasing absorbance readings by almost 300% at 5×10^{-4}M. As would be expected from Figure 4.13, NDGA proved to be the most potent inhibitor of phenol red oxidation, inhibiting absorbance readings by almost 80% at 10^{-6}M and by 20% at 10^{-7}M.

In all cases, addition of catalase inhibited totally any phenol red

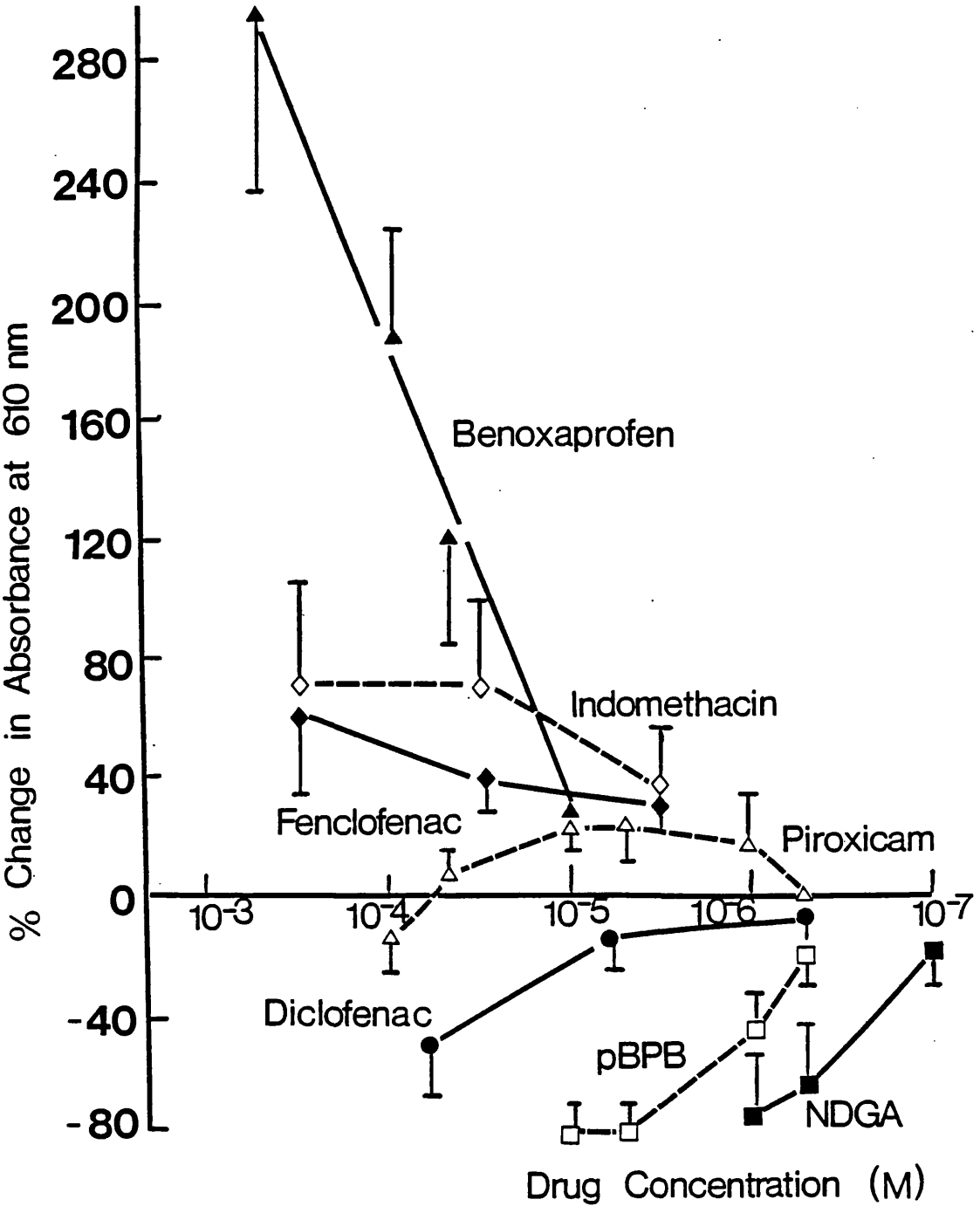


Figure 4.16 % change in absorbance readings at 610nm due to presence of drugs with 2 x 10^6 resting neutrophils. Results expressed $\bar{X}$ ± SD of 3 experiments.

oxidation, proving that resting cells produce only H_2O_2 and not ROOH and that the inhibitors only stimulated additional release of H_2O_2 and not ROOH.

4.4 Ferrithiocyanate Assay

This assay for H_2O_2 was used in comparative experiments with the phenol red assay. Using both assays and neutrophils from the same individuals, resting cell levels of H_2O_2 production and HAGG-stimulated levels of H_2O_2 production after 30 minutes incubation at 37°C were measured. These always correlated extremely well. The results from one such experiment are shown in Table 4.7. This demonstrates that the ferrithiocyanate assay measures only H_2O_2 production as catalase inhibits completely any response and the presumed H_2O_2 production in the phenol red assay i.e. the catalase sensitive portion of oxidation correlates very well with the H_2O_2 measured in the ferrithiocyanate assay.

In order to determine why some inhibitors were directly affecting the phenol red assay, they were tested in the ferrithiocyanate assay. Unfortunately the inhibitors which were not water-soluble and had been dissolved in DMSO still could not be tested as the presence of 1% v/v DMSO itself affected this assay. Ethanol and methanol had the same effect and so could not be used as solvents for these particular inhibitors. However, the water-soluble inhibitors diclofenac sodium, quinacrine and BW 755C could all be tested.

It was found that none of the concentrations of diclofenac (6×10^{-4}M - 6×10^{-7}M) or quinacrine (10^{-4}M - 10^{-5}M) had any direct effect on this assay. Although BW 755C still had a direct inhibitory effect on the assay, it was to a much smaller extent than on the phenol red assay. This proves that none of these inhibitors directly affect the

Incubation (2×10^6/ml)	Phenol Red Assay-nmoles oxidized phenol red	Ferrithiocyanate Assay nmoles H_2O_2
Resting cells	5	5.5
Resting cells + 1500U catalase	0	0
Cells + 200µg HAGG	17.5	8
Cells + 200µg HAGG + 1500U catalase	8.5	0
Catalase-sensitive response to HAGG	9	8

Table 4.7: Resting cells and stimulated cells incubated in both the phenol red and ferrithiocyanate assays.

assay by breaking down H_2O_2, otherwise it would be expected that they would affect both assays to the same extent. The two remaining inhibitors which directly affected the phenol red assay, but which were not water-soluble, were pirprofen and NDGA. The relevant concentrations of these inhibitors were dissolved in the TCA used to acidify the ferrithiocyanate assay and were tested in the assay in this way. Neither proved to have any effect on the assay.

To investigate further the nature of the interaction of diclofenac and pirprofen with the phenol red assay, these inhibitors were incubated with 8.5 Units/ml peroxidase in the ferrithiocyanate assay for 10 minutes at 37°C in the presence of H_2O_2. This was in order to determine whether they interfered with the phenol red assay by inhibiting peroxidase. If this was the case one would expect that the peroxidase would fail to break down H_2O_2 in the presence of the inhibitors and thus more H_2O_2 would be detected in the assay than when peroxidase but no inhibitor was present. It appeared that, in fact, both inhibitors acted as an alternative substrate for peroxidase and were oxidized themselves by the enzyme in the presence of H_2O_2. Virtually no H_2O_2 was detected following incubation and yellow precipitates were formed in the reactions containing inhibitor, H_2O_2 and peroxidase.

4.5 Effects of Inhibitors on Enzymatically-Generated Superoxide

All the inhibitors were tested to determine whether they were capable of scavenging superoxide anion, thereby preventing its dismutation to H_2O_2 and explaining their ability to inhibit H_2O_2 production. Using the microassay technique described in Chapter 2, xanthine and xanthine oxidase were used to generate superoxide anion, detected by the reduction of cytochrome C, in the presence of a range of concentrations of

Drug	Concentration (M)	% of reduction of cytochrome C in absence of drug $\bar{X} \pm SD$ n = 6
Diclofenac	6.3×10^{-4}	52 ± 9
	6.3×10^{-5}	68 ± 17
	6.3×10^{-6}	80 ± 15
	6.3×10^{-7}	88 ± 11
Indomethacin	3×10^{-4}	61 ± 25
	3×10^{-5}	85 ± 13
	3×10^{-6}	88 ± 14
	3×10^{-7}	86 ± 10
Fenclofenac	3×10^{-4}	78 ± 18
	3×10^{-5}	89 ± 11
	3×10^{-6}	97 ± 3
Piroxicam	10^{-4}	99 ± 8
	10^{-5}	107 ± 15
	10^{-6}	107 ± 16
Pirprofen	10^{-3}	65 ± 20
	10^{-4}	87 ± 14
	10^{-5}	93 ± 11
	10^{-6}	91 ± 16
NDGA	5×10^{-6}	69 ± 14
	10^{-6}	86 ± 13
	5×10^{-7}	100 ± 1
	10^{-7}	95 ± 10
Benoxaprofen	5×10^{-4}	86 ± 16
	10^{-4}	76 ± 10
	5×10^{-5}	86 ± 13
	10^{-5}	93 ± 11

Drug	Concentration (M)	% of reduction of cytochrome C in absence of drug $\bar{X} \pm SD$ n = 6
BW 755C	10^{-4}	80 ± 13
	10^{-5}	80 ± 11
	10^{-6}	72 ± 17
	10^{-7}	78 ± 19
pBPB	10^{-5}	83 ± 19
	5×10^{-6}	85 ± 16
	10^{-6}	90 ± 13
	5×10^{-7}	89 ± 7

Table 4.8 The effect of drugs on enzymatically-produced superoxide

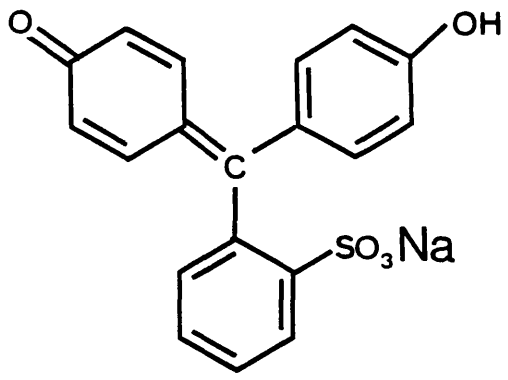
inhibitors. The results are shown in Table 4.8 and are expressed as a % of the reduction of cytochrome C in the absence of any inhibitor. The results indicate that the highest concentrations of diclofenac sodium, indomethacin, fenclofenac, pirprofen and NDGA may scavenge superoxide, although the possibility that they may in fact be inhibiting xanthine oxidase cannot be ruled out. However, more importantly, any slight scavenging effects these drugs are having does not explain the much larger inhibitory effects that NDGA and pBPB have on H_2O_2 production by neutrophils (Figure 4.13) and do not interfere with the ability of fenclofenac, indomethacin and benoxaprofen to stimulate H_2O_2 production by resting neutrophils.

Structures of the Inhibitors

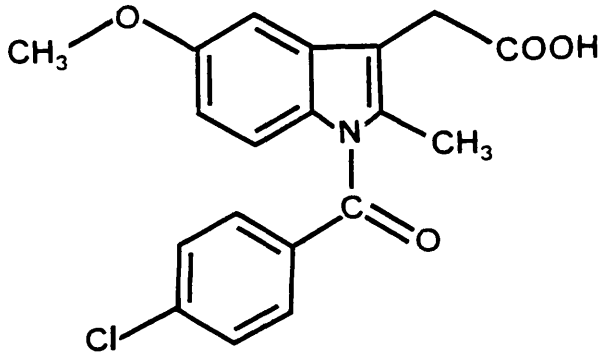
For comparative purposes, the molecular structures of the inhibitors used during the course of this work, together with that of phenol red, are shown in Figures 4.17 and 4.18.

The Effects of Piroxicam and Diclofenac on Neutrophil Activity In Vivo

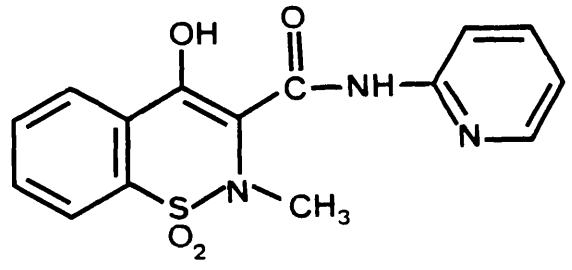
In order to determine whether or not either of these NSAIDS had any effect on neutrophil activity in vivo, neutrophils were tested from patients before and after receiving either piroxicam 20mg daily or diclofenac 100mg in a slow-release preparation daily for four weeks. A total of 14 patients were studied, 3 male and 11 female, age range 35 - 75. Three of the patients had early synovitis and the remainder had osteoarthritis. Four of the osteoarthritis patients received both piroxicam and diclofenac, separated by one week's washout with paracetamol. The remaining patients were tested before and after one drug only. Blood samples were taken following one week's treatment with



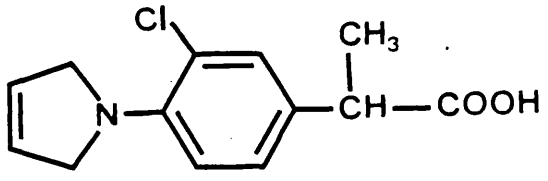
Phenol Red



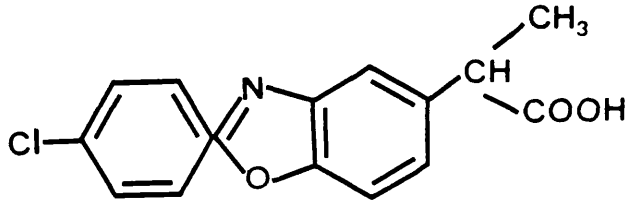
Indomethacin



Piroxicam

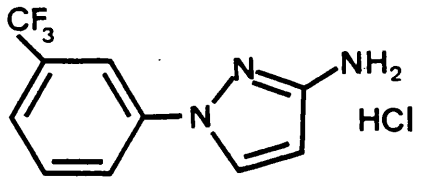


Pirprofen

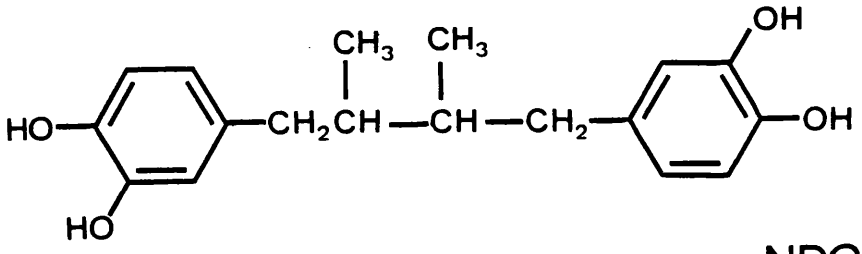


Benoxaprofen

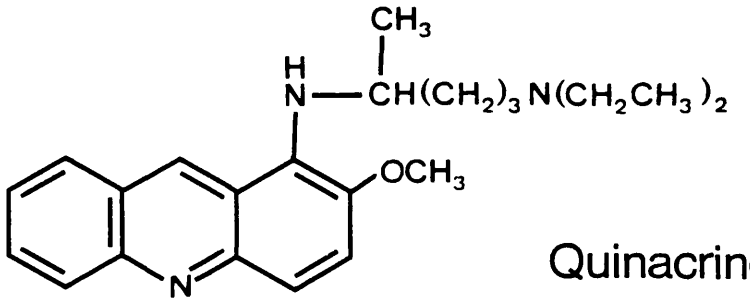
Figure 4.17 Molecular structures of compounds used in this study.



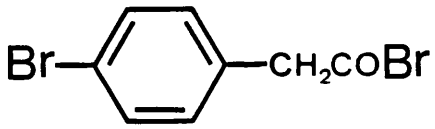
BW 755C



NDGA



Quinacrine



pBPB

Figure 4.18 Molecular structures of compounds used in this study.

paracetamol and then on the last day of four weeks treatment with either diclofenac or piroxicam. The osteoarthritis patients formed part of a double-blind study and thus individual treatment was unknown until after completion of this study. It was known that the early synovitis patients were receiving piroxicam.

Levels of H_2O_2 and ROOH secretion from 2 x 10^6/ml neutrophils were measured using the phenol red assay following incubation at 37°C for 30 minutes. Cells were either unstimulated or else stimulated with HAGG (200µg/ml) or FMLP (20µM). Figures 4.19 - 4.22 show the results from this study. Lines connect the results for an individual before taking the drug and then after 4 weeks drug treatment. It is obvious from all these figures that neither piroxicam nor diclofenac affect H_2O_2 or ROOH production by unstimulated neutrophils or by those stimulated by either HAGG or FMLP. An attempt was made to correlate efficacy of drug with effect on neutrophil activity. The osteoarthritis patients had been asked to assess efficacy by scoring the drug as 1 - excellent down to 4 - poor and by estimating a pain score (visual analogue scale) judged by morning and evening pain and pain at rest. However, no correlation was found between the apparent effectiveness of either drug based on these criteria and its effects on neutrophil activity.

Reproducibility of the Phenol Red Assay

The validity of comparing neutrophil activation in the same individual over a time period can be assessed by testing the reproducibility of the assay. This may be done by examining the levels of H_2O_2 and ROOH produced by stimulated neutrophils from the same individual on different occasions. The amounts of ROOH produced in response to HAGG and H_2O_2 produced in response to FMLP is expressed as a % of the amounts produced

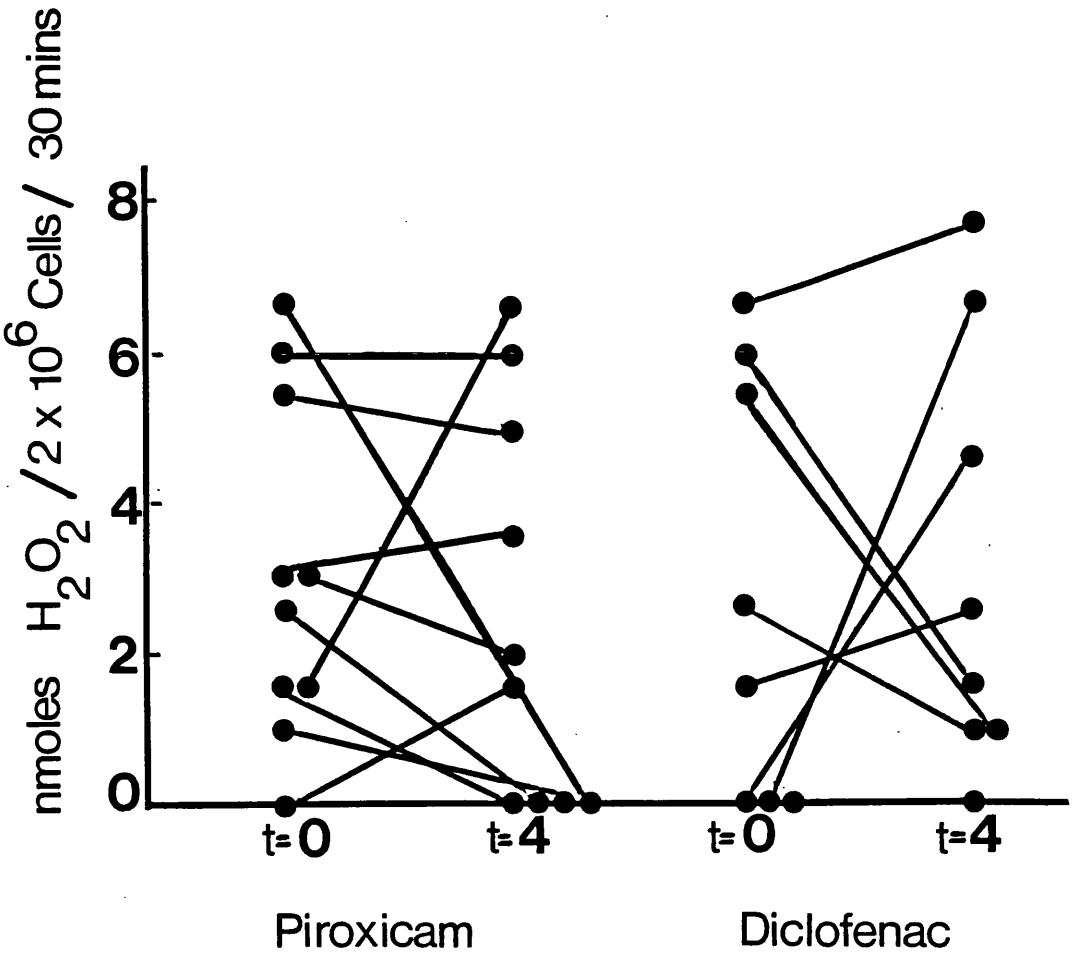


Figure 4.19 H_2O_2 production by unstimulated neutrophils from patients taking piroxicam 20mg od and diclofenac 100mg od for 4 weeks. Lines connect the results for an individual before taking the drug (t=0) and then after 4 weeks drug treatment (t=4).

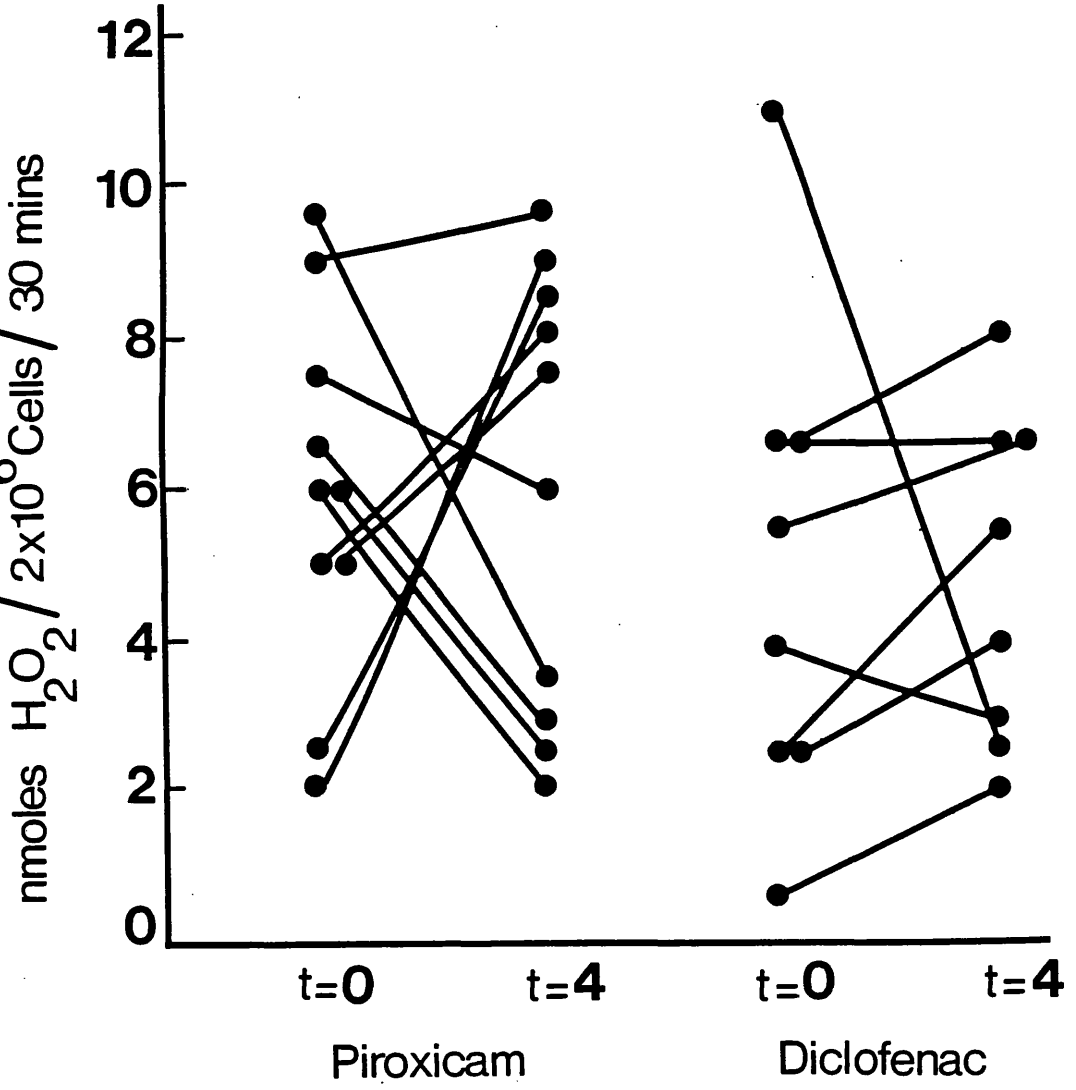


Figure 4.20 H_2O_2 production by HAGG (200µg)-stimulated neutrophils from patients taking piroxicam 20mg od and diclofenac 100mg od for 4 weeks. Lines connect the results for an individual before taking the drug (t=0) and then after 4 weeks drug treatment (t=4).

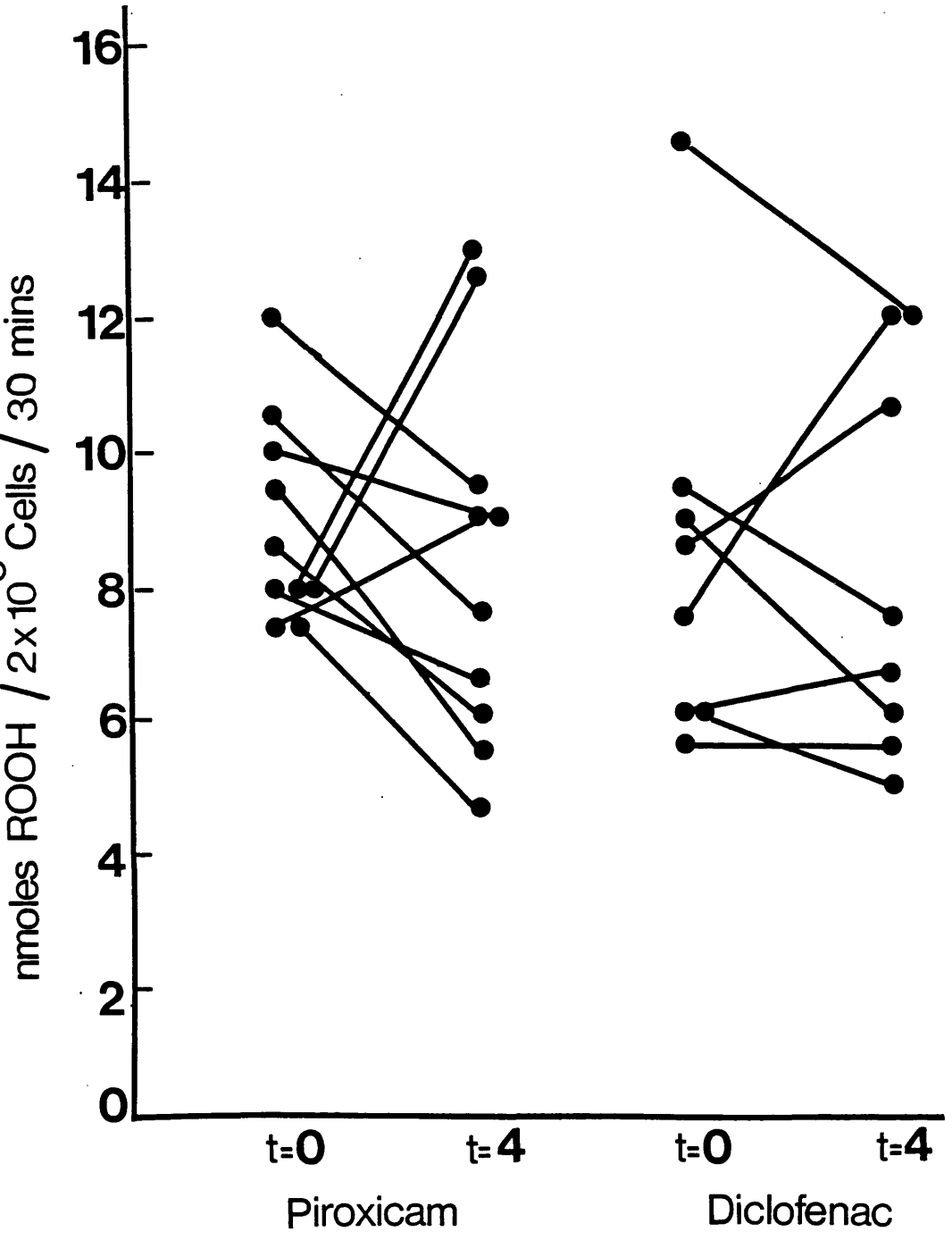


Figure 4.21 ROOH production by HAGG (200µg)-stimulated neutrophils from patients taking piroxicam 20mg od and diclofenac 100mg od for 4 weeks. Lines connect the results for an individual before taking the drug (t=0) and then after 4 weeks drug treatment (t=4).

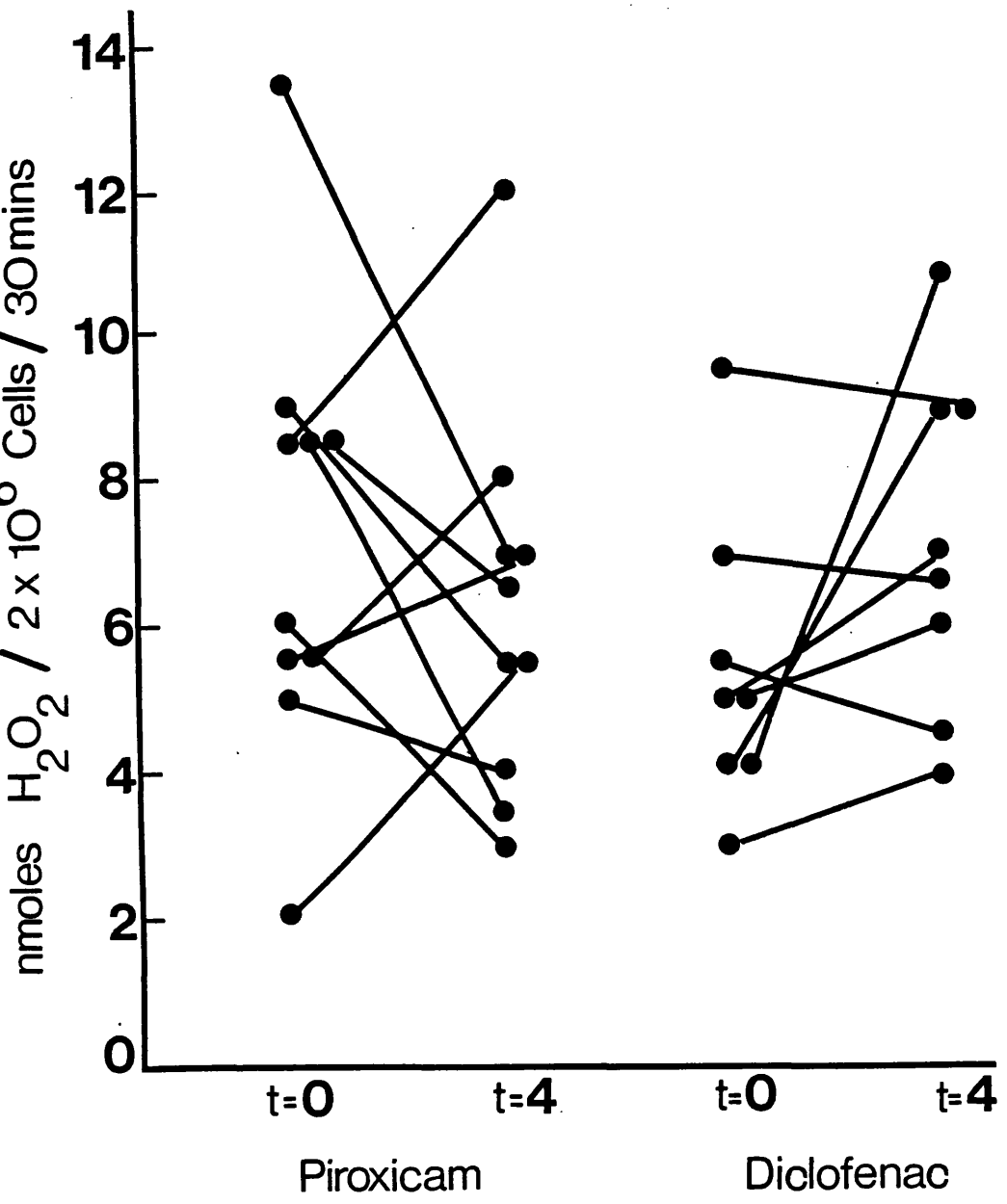


Figure 4.22 H_2O_2 production by FMLP (20μM)-stimulated neutrophils from patients taking piroxicam 20mg od and diclofenac 100mg od for 4 weeks. Lines connect the results for an individual before taking the drug (t=0) and then after 4 weeks drug treatment (t=4).

when the individual was first tested. The mean ± SD of these %s can be taken and the nearer this is to 100%, the more reproducible the assay. Studying 12 individuals whose neutrophils had been stimulated with HAGG to produce ROOH on more than one occasion resulted in a mean value of % original ROOH production of 83 ± 35%. Studying 6 individuals whose neutrophils had been stimulated with FMLP to produce H_2O_2 on more than one occasion resulted in a mean value of % original H_2O_2 production of 91 ± 28%. Certainly a range of 2 SDs from these figures would encompass the % change in stimulated levels of ROOH or H_2O_2 production seen in the 4 patients tested after receiving both diclofenac and piroxicam. The large standard deviations may be explained because the absolute values are small (usually less than 10nmoles) and thus % changes tend to be artificially large.

CHAPTER FIVE

RESULTS C

5. INVESTIGATION INTO THE OXIDATIVE METABOLISM OF NEUTROPHILS FROM PATIENTS WITH PROGRESSIVE SYSTEMIC SCLEROSIS (PSS)

It has been suggested in the Introduction to this thesis that it is possible that oxygen-derived free radicals released from phagocytic cells could be implicated in certain features of PSS including damage to vascular endothelium, abnormal function of serum protease inhibitors and increased frequency of chromosome breakage. Accordingly, H_2O_2 and ROOH production was determined using the phenol red assay in 16 patients with PSS, 16 healthy volunteers, 15 patients with RA and 8 patients with peripheral vascular disease. Measurements were made on unstimulated neutrophils and following incubation with HAGG or with FMLP for 30 minutes at 37°C.

PSS Patients

16 patients were studied, 15 females and 1 male, age-range 29 - 82 years. There were 5 patients receiving penicillamine 3 receiving NSAIDS, 3 receiving colchicine, 1 receiving prednisolone and 5 receiving no therapy. 10 patients had localized disease, a limited form of PSS with features to satisfy the criteria for the CREST variant of the disease and 6 patients had diffuse scleroderma, as assessed by Dr. P. J. Maddison.

Healthy Volunteers

16 healthy volunteers were studied, 15 females and 1 male, age range 26 - 73 years. One volunteer was receiving treatment with indomethacin. Experiments using neutrophils from PSS patients and healthy volunteers were performed at the same time in age-and sex-matched pairs.

Rheumatoid Arthritis Patients

15 patients were studied, 13 females and 2 males, age-range 37 - 74 years. There were 5 patients receiving penicillamine, 9 receiving NSAIDS, 1 receiving prednisolone and 3 receiving no therapy. They all had active joint disease at the time of the study, but none had evidence of systemic features such as vasculitis.

Peripheral Vascular Disease Patients

8 patients were studied with atherosclerosis, 5 females and 3 males, age-range 56 - 87 years. There were 5 patients receiving diuretics, 1 receiving prednisolone and 1 receiving hypoglycaemic therapy.

H_2O_2 production in 30 minutes by 1 x 10^6 unstimulated neutrophils for all four groups is shown in Figure 5.1. Unstimulated cells from PSS patients produced significantly more H_2O_2 than those of any other group ($p<0.05$). Moreover, in 12/16 cases, on a given day, the PSS patient's neutrophils produced more H_2O_2 than the healthy control's neutrophils. 3 matched pairs produced virtually the same amount and in only 1 pair did the healthy control's neutrophils produce more H_2O_2 than the PSS patient's neutrophils. This matched pair data is shown in Figure 5.2.

ROOH production in 30 minutes by 1 x 10^6 neutrophils stimulated by HAGG for all four groups is shown in Figure 5.3. Neutrophils from PSS patients generated significantly more ROOH than did the control groups ($p<0.005$). RA patients' neutrophils produced significantly less ROOH than healthy controls ($p<0.05$). H_2O_2 production over and above unstimulated cell levels in response to HAGG was not studied. In Chapter 3, Figure 3.15 showed that when 1 x 10^6 neutrophils are stimulated by HAGG only 8% of the total phenol red oxidation is due to

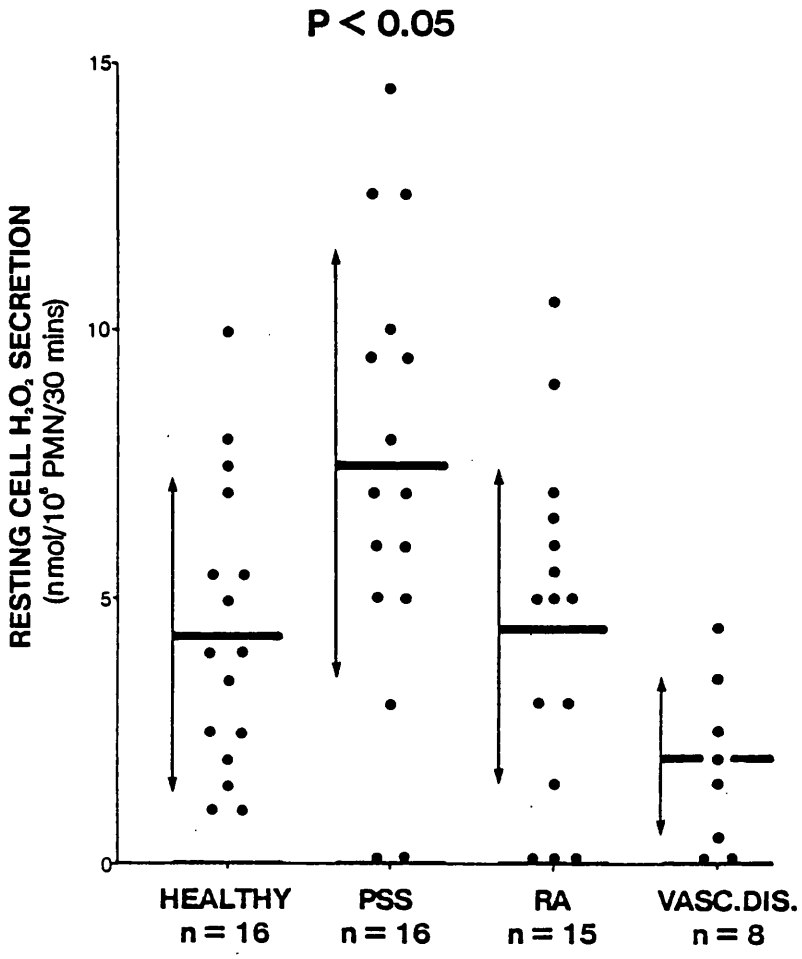


Figure 5.1 H_2O_2 secreted by unstimulated cells (nmol/10^6 neutrophils/30 minutes) from healthy volunteers and patients with PSS, RA and peripheral vascular disease.

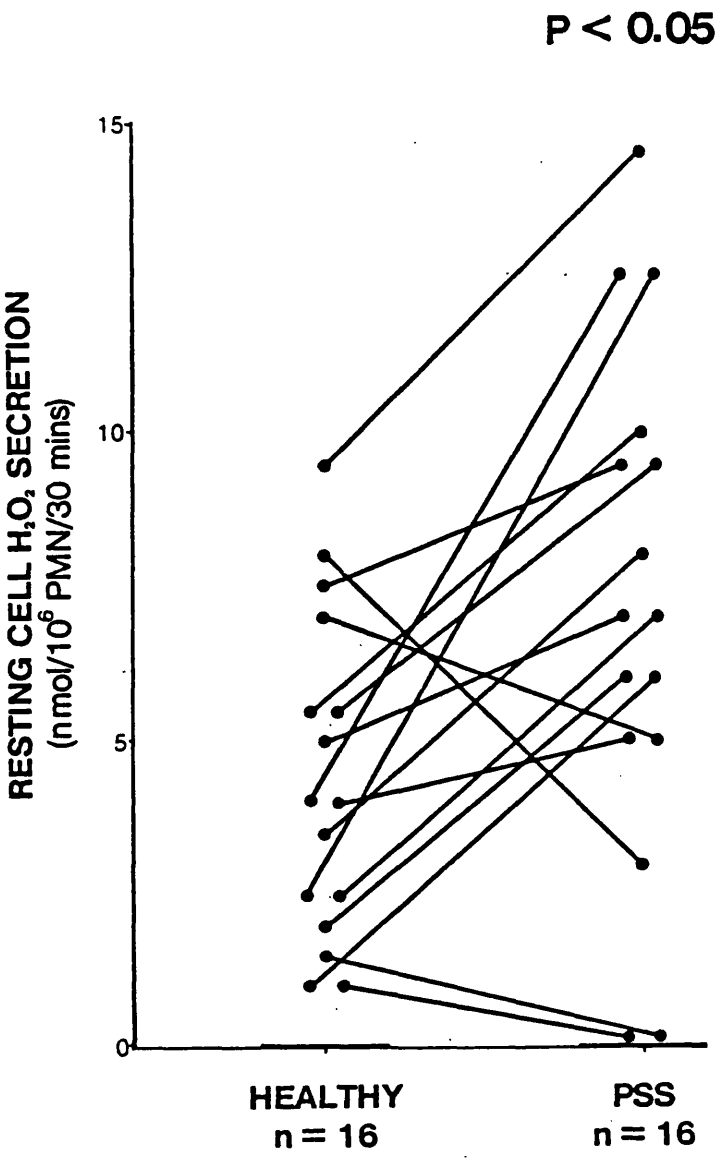


Figure 5.2 H_2O_2 secreted by matched pairs of healthy and PSS unstimulated neutrophils.

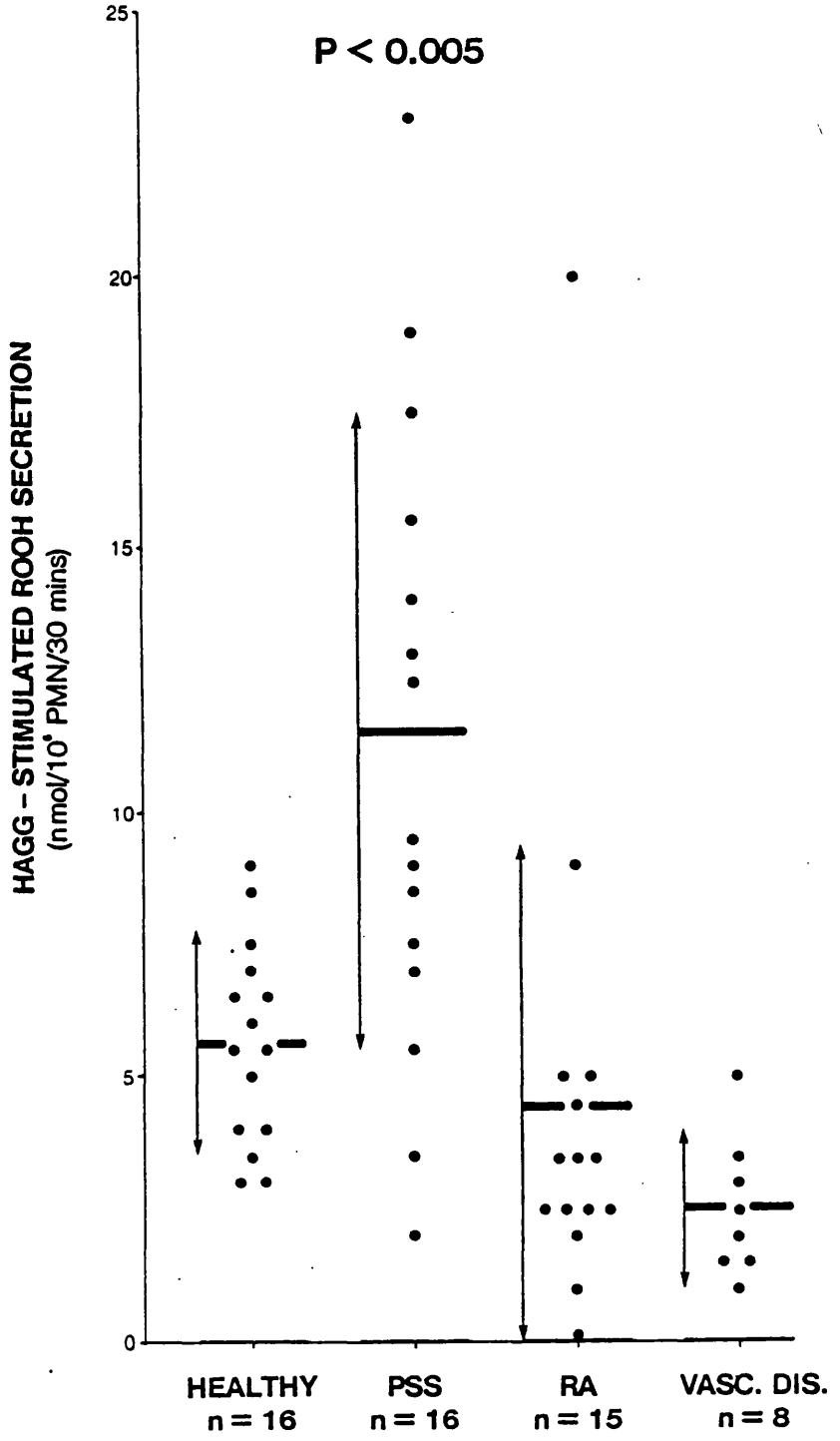


Figure 5.3 ROOH secreted by neutrophils stimulated by 200μg HAGG (nmol/10^6 neutrophils/30 minutes) from healthy volunteers and patients with PSS, RA and peripheral vascular disease.

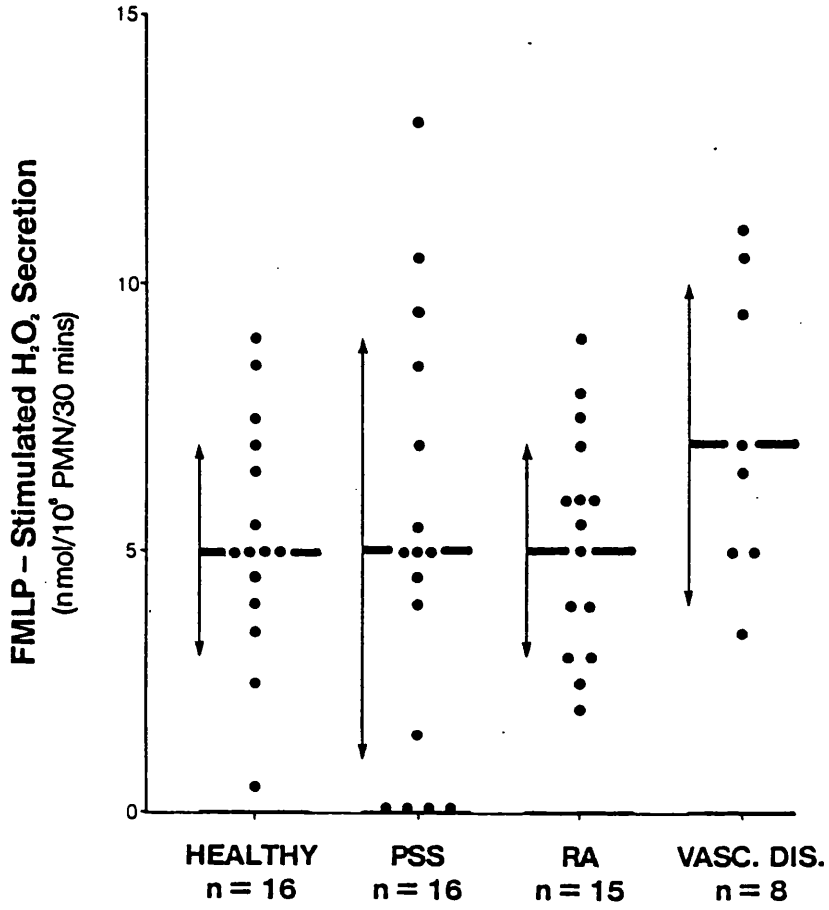


Figure 5.4 H_2O_2 secreted by neutrophils stimulated by FMLP (20μM) (nmol/10^6 neutrophils/30 minutes) from healthy volunteers and patients with PSS, RA and peripheral vascular disease.

H_2O_2, and that 92% is due to ROOH. Thus the absolute amounts of H_2O_2 produced in these experiments were very small. In contrast, it can be seen in Figure 5.4 that following stimulation with FMLP, there was no significant difference between any of the groups in the amount of H_2O_2 generated in 30 minutes by 1 x 10^6 neutrophils above unstimulated levels. As the enhanced neutrophil activity appeared to be detectable with HAGG as the stimulus, but not with FMLP, it was decided to study the Fc receptors on the neutrophils from PSS patients, employing an EA rosetting technique described in Chapter 2. Figure 5.5 shows the % rosetting cells from healthy controls and from patients with PSS at various dilutions of IgG on a log scale. There is a significant shift to the right of the line for patients' cells ($p<0.01$ at 1/500 and 1/750 dilutions). This shift is by a factor of approximately 2. Moreover, if levels of % rosetting cells at 1/500 dilution of IgG for a particular individual, are plotted against the amount of H_2O_2 produced in 30 minutes by 1 x 10^6 unstimulated cells from that same individual, there is a strong positive correlation, with a value of the correlation coefficient r, of 0.93 (Figure 5.6). In addition, plotting the % rosetting cells at 1/500 dilution of IgG against the amount of ROOH produced by HAGG-stimulated neutrophils from the same individuals, also gives a strong positive correlation, with a value of the correlation coefficient r, of 0.85 (Figure 5.7).

Correlation with Disease Activity

The mean values for H_2O_2 production by unstimulated cells and ROOH production by HAGG-stimulated cells for the localized and diffuse types of the disease, both active and inactive are shown together with the equivalent values for healthy control cells in Table 5.1.

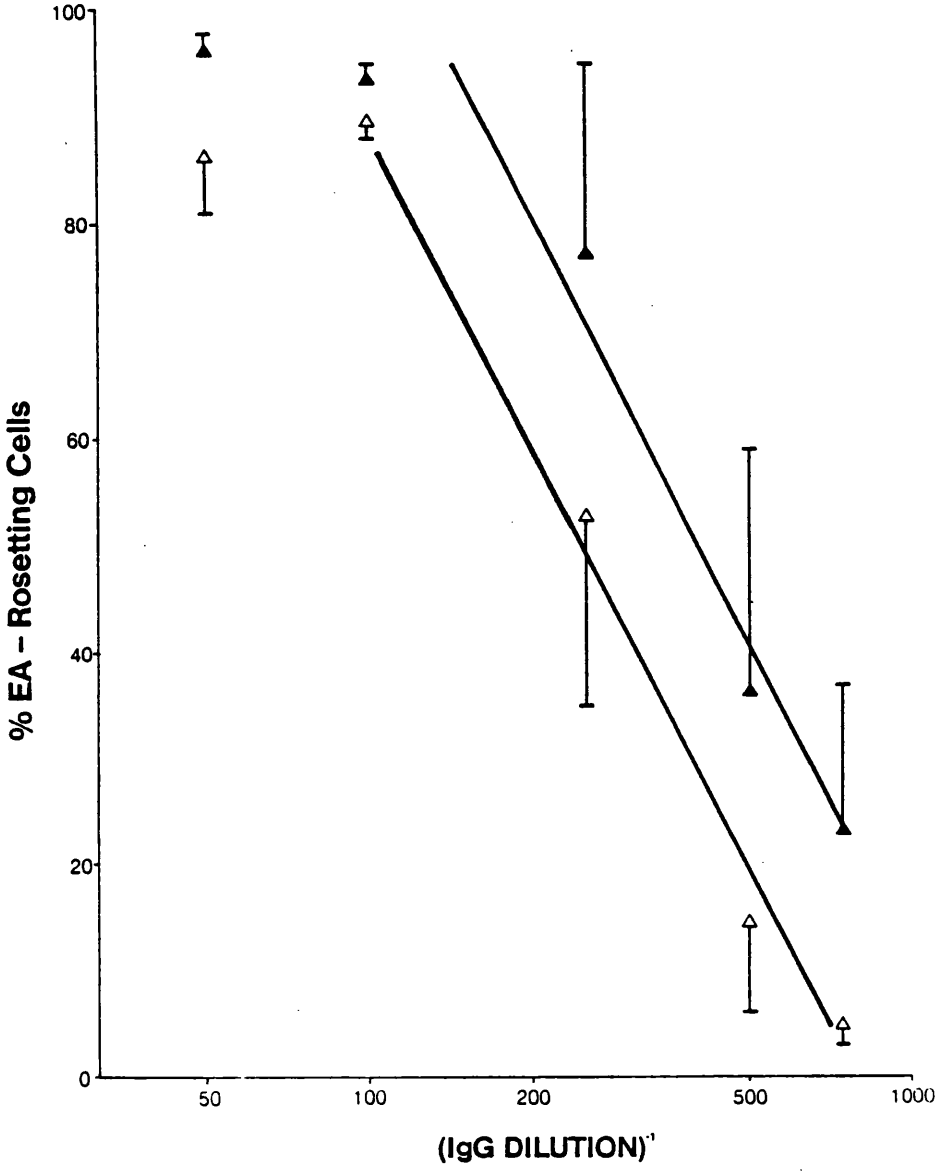


Figure 5.5 % EA-Rosetting cells from △ healthy controls and ▲ PSS patients at various dilutions of IgG.

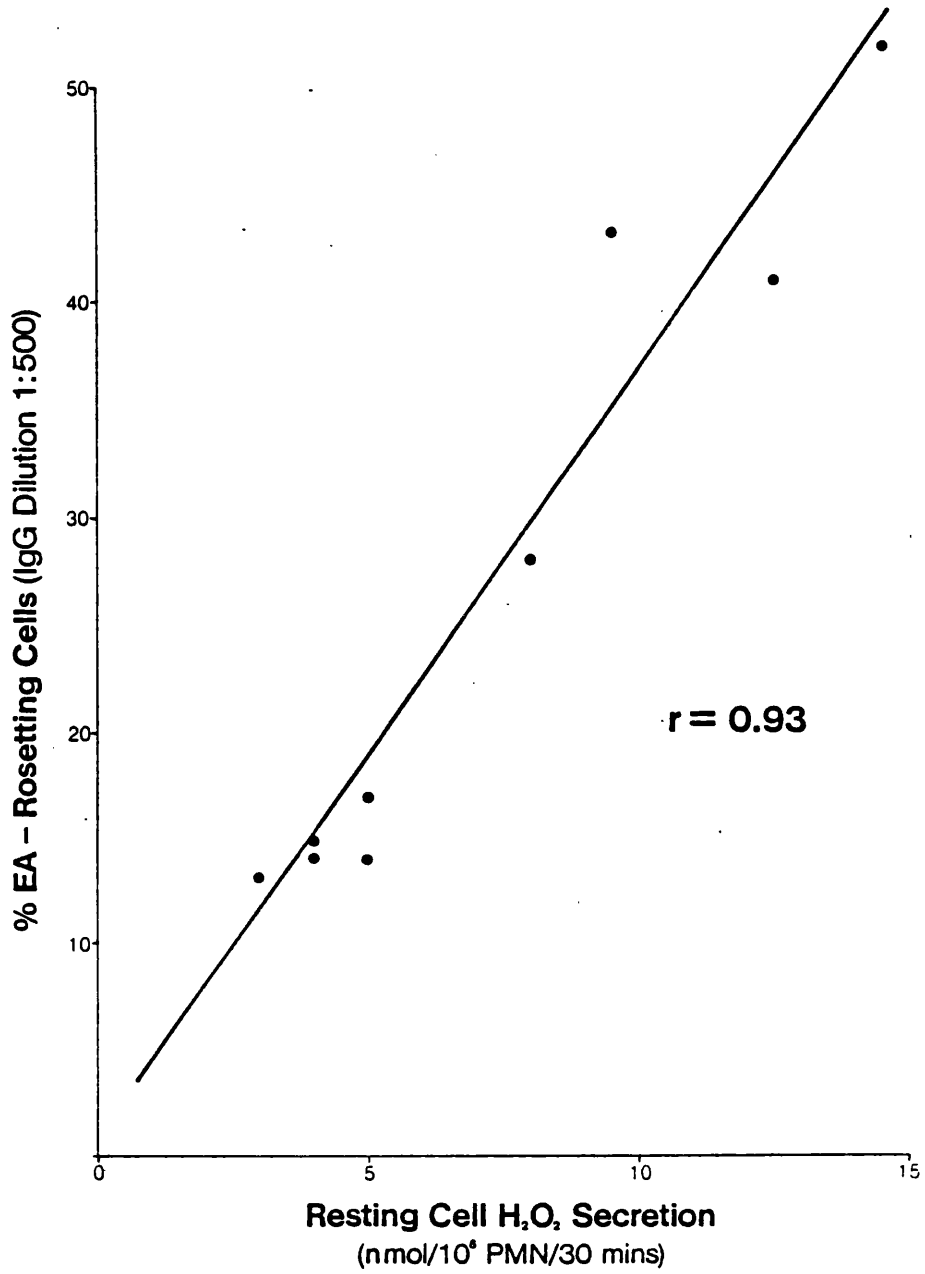


Figure 5.6 Correlation between % EA-Rosetting cells at IgG dilution $^1/500$ and unstimulated neutrophil H_2O_2 secretion (nmol/10^6 neutrophils/30 minutes).

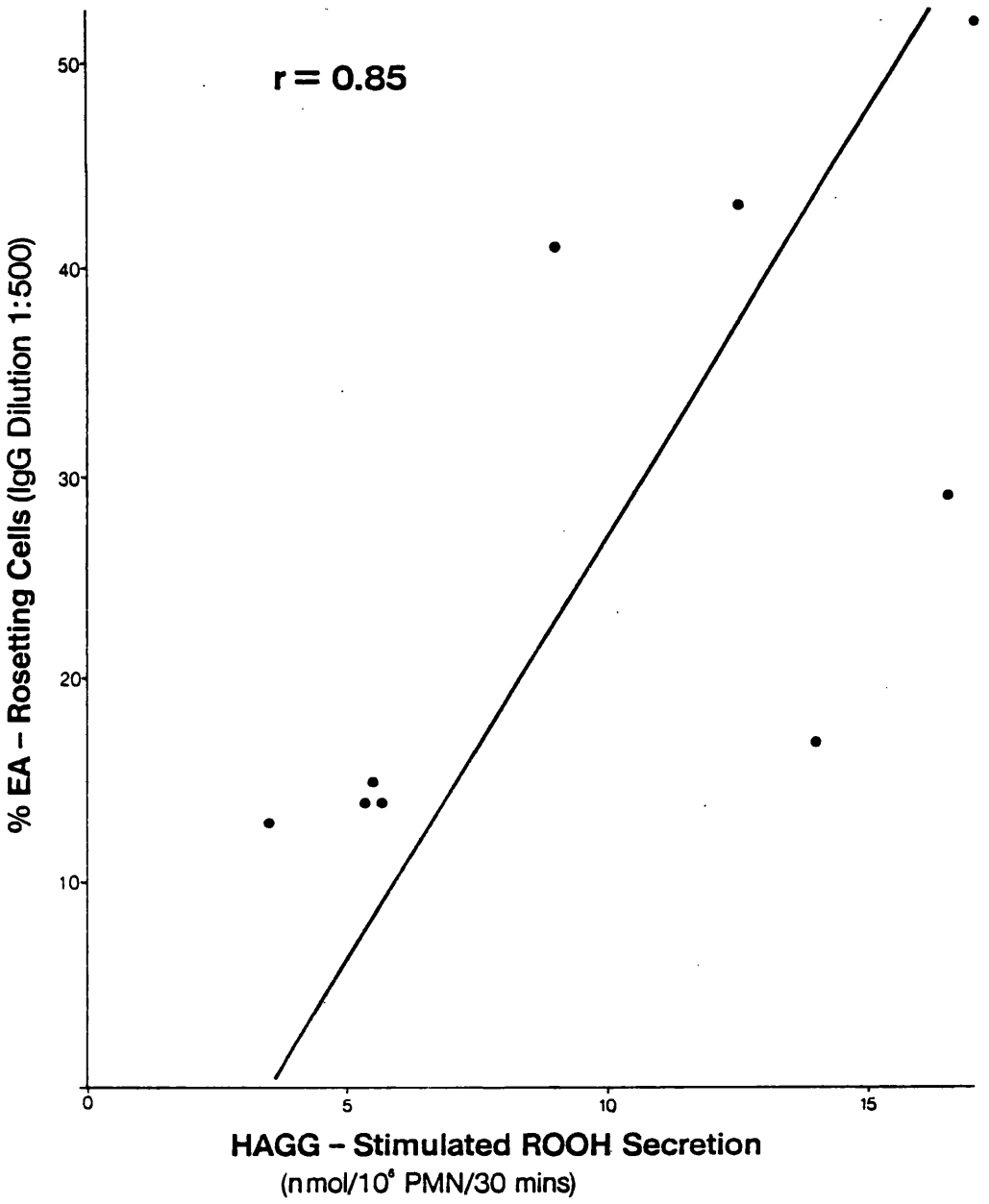


Figure 5.7 Correlation between % EA-rosetting cells at IgG dilution $^{1}/500$ and 200μg HAGG-stimulated ROOH secretion (nmol/10^{6} neutrophils/30 minutes).

Peroxide	Patient Group				
	CREST Active n = 4	Inactive n = 6	Diffuse Active n = 4	Inactive n = 2	Normal n = 16
H_2O_2	10.5 ± 3	7.6 ± 3	4.5 ± 4	5 ± 6	4.3 ± 2.5
ROOH	12.5 ± 5	12 ± 3	7.5 ± 7	16 ± 10	5.6 ± 3

Table 5.1 Mean values (± SD) of H_2O_2 production by unstimulated cells and ROOH production by HAGG-stimulated cells in the different disease groups within the PSS patient group compared with those for normal cells.

There is a significant difference between H_2O_2 production by unstimulated cells from both active ($p<0.01$) and inactive ($p<0.05$) CREST patients compared with healthy controls, and also between ROOH production by HAGG-stimulated cells from both active ($p<0.01$) and inactive ($p<0.01$) CREST patients compared with healthy controls. There is no significant difference between H_2O_2 production or ROOH production by unstimulated cells or HAGG-stimulated cells from both active and inactive diffuse disease patients compared with healthy controls. However, there is no significant difference between CREST and diffuse forms of disease in either H_2O_2 or ROOH production, comparing them both as whole groups or divided into active and inactive disease. This is presumably due to the small numbers involved. Thus it seems that the significant difference seen between the whole PSS group and controls for H_2O_2 and ROOH production is due to those patients in the group with CREST rather than diffuse disease.

CHAPTER SIX

DISCUSSION

D I S C U S S I O N

When human phagocytic cells are activated, they respond by producing oxygen-derived free radicals; $O_2^{\bullet-}$ and H_2O_2 and also another peroxide, designated ROOH. The peroxides can be measured by the oxidation of phenol red in a peroxidase-dependent reaction and $O_2^{\bullet-}$ can be measured by the reduction of cytochrome C. The H_2O_2 produced can also be detected, although not quantitatively, by its ability to oxidize serum SH groups. The increase in oxygen-uptake by these cells which occurs upon activation can also be determined using an oxygen electrode.

The major product of oxidative metabolism and its absolute amount varies with the type of cell and the stimulus employed, both in terms of its structure and concentration. The stimulus also determines the kinetics of generation of the products, also depending upon its concentration. Unstimulated neutrophils secrete only $O_2^{\bullet-}/H_2O_2$. Those stimulated with FMLP produce mainly $O_2^{\bullet-}/H_2O_2$ with only minimal amounts of ROOH. However, HAGG- and opsonized zymosan-stimulated neutrophils secrete mainly ROOH with very little $O_2^{\bullet-}/H_2O_2$ being generated. The proportion of ROOH produced in response to HAGG is greater at low cell numbers. This pattern is repeated with monocytes, except that these cells secrete a higher proportion of H_2O_2 than ROOH compared with neutrophils in response to HAGG and opsonized zymosan. However, neutrophils generate more H_2O_2 in absolute terms than do monocytes in response to all stimuli.

The results shown in Chapter 3 of this thesis indicate that all the H_2O_2 that is produced in response to a given stimulus is generated in the first few minutes of incubation and thereafter no more is produced. In contrast ROOH is produced after an initial lag period and continues to be produced at the same rate throughout the incubation period. This can be supported further by reference to the results for overall

peroxide production by the individual stimuli. FMLP-stimulated neutrophil production of peroxide (all H_2O_2) occurs at a faster rate than that following opsonized zymosan-stimulated production of peroxide (mainly ROOH). Neutrophils stimulated with low concentrations of HAGG produce more H_2O_2 than ROOH, but this production falls at higher concentrations of HAGG, whereas ROOH production continues to increase. Similarly, $O_2^{\bullet-}$ production falls at higher concentrations of HAGG. This may be due to scavenging of $O_2^{\bullet-}$ by HAGG before it can be detected by reducing cytochrome C.

Comparing absolute amounts of oxygen consumed, $O_2^{\bullet-}$ generated and subsequent H_2O_2 production, there are apparent discrepancies in the values which could be predicted from the equations:

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^{\bullet-} + NADP^+ + H^+$$

$$2O_2^{\bullet-} + 2H^+ \xrightarrow[\text{or SOD}]{\text{spontaneous}} H_2O_2 + O_2$$

It can be deduced from these equations that 1nmole of oxygen yields 1nmole $O_2^{\bullet-}$ and 2nmoles $O_2^{\bullet-}$ yield 1nmole H_2O_2. However, results shown in Chapter 3 indicate that 2×10^6 neutrophils stimulated with 200µg/ml HAGG consume, in 10 minutes, 90nmoles oxygen, yet produce only 1.5nmoles $O_2^{\bullet-}$ and 4.5nmoles H_2O_2. It is obvious that insufficient superoxide is being detected to account for the H_2O_2 measured. This could be because it is dismutating into H_2O_2 before it can reduce the cytochrome C; reacting with/scavenged by HAGG before it can reduce the cytochrome C; or else it is remaining bound to the enzyme system and hence it is unavailable to reduce cytochrome C. Also from these equations it can be seen that ten times more oxygen is being consumed than is required to produce 4.5nmoles H_2O_2. As virtually all the

oxygen consumed by the neutrophil goes toward oxygen-derived free radical production, this suggests that 90% of the H_2O_2 generated is not secreted. However, this amount is not detected even in lysed cells presumably because it is destroyed intracellularly by glutathione peroxidase, catalase or myeloperoxidase. It is interesting to note that Root et al (1975) found that, in response to a phagocytic stimulus, oxygen consumption by neutrophils was approximately 64nmoles, whereas H_2O_2 production was only 4 nmoles i.e. eight times more oxygen was being consumed than is required to produce 4nmoles H_2O_2 - not dissimilar to the results presented here. Moreover, if the oxygen regenerated by superoxide dismutase is taken into account, this means that sixteen times more oxygen was being consumed than is required to produce 4nmoles H_2O_2. However, this is only a valid consideration if the dismutation occurs extracellularly and the oxygen is not produced intracellularly. When Root and Metcalf (1977) added 1mM azide to inhibit catalase and myeloperoxidase, apparent H_2O_2 release doubled and the molar relationships for oxygen consumed, $O_2^{\bullet-}$ and H_2O_2 release were found to be 1.00: 0.34: 0.51. Furthermore, by using cytochalasin B-treated cells an agent that inhibits phagocytosis whilst permitting oxidative metabolism to occur, they were able to show that all the oxygen taken up during the respiratory burst was converted to H_2O_2 by dismutation and that this dismutation reaction was the only important source of H_2O_2 generated during the burst.

The use of inhibitors of arachidonic acid metabolism helps to elucidate the possible identity of ROOH. From the results shown in Chapter 4 it can be seen that the most potent inhibitors of ROOH production are the phospholipase A_2 inhibitor pBPB and the lipoxygenase inhibitor NDGA. Cyclooxygenase inhibitors are comparatively inactive. This would support the conclusion that the unknown peroxide is a product of arachidonic acid metabolism (due to the potent inhibition by pBPB)

and moreover that it is a product of the lipoxygenase pathway, as NDGA is as potent an inhibitor as pBPB. It has already been discussed in the Introduction that human neutrophils metabolize arachidonic acid mainly via the 5-lipoxygenase pathway and very little is metabolized via the cyclooxygenase pathway. As the oxidant of phenol red is undoubtedly a peroxide, because it is a peroxidase-dependent reaction, this means that ROOH is most likely to be the 5-hydroperoxy derivative of arachidonic acid, 5-HPETE. The fact that monocytes produce a higher proportion of H_2O_2 than ROOH compared with neutrophils indicates that they either have less lipoxygenase enzyme than neutrophils or that they metabolize more arachidonic acid via the cyclooxygenase pathway than do neutrophils. It would have been more satisfactory to have performed experiments attempting to detect LTB_4 to prove this hypothesis more conclusively. This would have involved the use of radio-labelled exogenous arachidonic acid for neutrophils to metabolize (and there is no guarantee that exogenous arachidonic acid would give the same products as endogenous arachidonic acid) and the subsequent isolation of labelled LTB_4 using thin layer chromatography techniques. If ROOH is 5-HPETE one might expect the mixed cyclooxygenase/lipoxygenase inhibitor benoxaprofen to prove more potent in inhibiting ROOH release than in fact it is. However, it may be that benoxaprofen is more potent as a cyclooxygenase inhibitor than a lipoxygenase inhibitor. In fact the concentrations used by Harvey et al (1983), using guinea-pig peritoneal cells as a source of 5-lipoxygenase, to inhibit production of 5-HETE, were 2×10^{-4}M (96% inhibition) to 3×10^{-5}M (61% inhibition). This compares with the results presented in this thesis of 97% inhibition of ROOH production at 10^{-3}M and 45% inhibition at 10^{-5}M. Thus the concentrations used to demonstrate its ability to inhibit 5-lipoxygenase are similar to those used in this work.

Benoxaprofen does inhibit ROOH production at concentrations found to inhibit 5-lipoxygenase. If it does not inhibit 12- and 15-lipoxygenases, as Harvey et al found (1983), then ROOH must be derived from the 5-lipoxygenase enzyme. Alternatively this might suggest that it is 15-HPETE rather than 5-HPETE that is being detected. However, as the amount of human neutrophil 15-lipoxygenase is very small compared with 5-lipoxygenase, this would seem unlikely (Naccache and Sha'afi, 1983).

The ability of HAGG and opsonized zymosan, but not apparently FMLP, to stimulate 5-HPETE production needs to be considered further. It could be that as different concentrations of HAGG stimulate neutrophils to produce the two peroxides in different proportions, different concentrations of FMLP or opsonized zymosan would also generate different proportions of products. For instance, a higher concentration of FMLP than 20μM may generate 5-HPETE as well as H_2O_2 and a lower concentration of opsonized zymosan than 800μg/ml may generate a higher proportion of H_2O_2. Thus there is no reason to suppose that FMLP does not stimulate either human monocytes or neutrophils to produce leukotrienes, nor to suppose that unstimulated cells do not produce leukotrienes. In fact, Ham et al (1983) have demonstrated that indeed FMLP does stimulate LTB_4 production, but in pmol/10^7 cells quantities. These amounts, detected using sensitive HPLC techniques, are obviously too small to be detected using the much cruder, less sensitive phenol red assay. However, Clancy, Dahinden and Hugli (1983) found that FMLP does not stimulate LTB_4 production by neutrophils from endogenous arachidonic acid, only via exogenous sources, and also that unstimulated cells do not produce LTB_4. Similarly, Palmer and Salmon (1983) were able to demonstrate LTB_4 release by human neutrophils in response

to opsonized zymosan but not in response to FMLP. Williams, Czop and Austen (1984) have also shown that human monocytes release quantitatively more leukotrienes following stimulation with opsonized zymosan than with IgG-coated erythrocytes. This also fits the pattern of ROOH release in this study. The ability of HAGG and opsonized zymosan but not FMLP to stimulate 5-HPETE release may be explained by considering the type of stimuli they are. The large, particulate, membrane-perturbing stimulus of HAGG or opsonized zymosan is going to be a much more powerful stimulus, involving stimulating every appropriate receptor on the neutrophil surface, than the soluble FMLP stimulus. Thus it is possible that stimulation of the NADPH oxidase does not need such a gross effect on the neutrophil as activation of phospholipase A_2. This is supported by the fact that low concentrations of HAGG stimulate mainly H_2O_2 production rather than 5-HPETE. It would have been interesting to use cytochalasin B-treated cells to see if the pattern of proportion of H_2O_2/5-HPETE released in response to the various stimuli changed. The lag-time associated with 5-HPETE production may indicate that in fact stimulation of phospholipase A_2 is dependent upon NADPH oxidase first being stimulated to produce $O_2^{\bullet-}$. However, it is not known, using the rather insensitive phenol red assay, whether this lag-time is genuine, or whether in the first 2 minutes, quantities of 5-HPETE too small to be detected are being produced. In any case, it is obvious that 5-HPETE production cannot be dependent upon H_2O_2 production as it is not prevented by catalase. Interaction of any stimulus with its receptor leads to a mobilization of intracellular calcium. This in turn is involved in activation of protein kinase C which changes the state of phosphorylation of an activator protein stimulating NADPH oxidase (Rink, Sanchez and Hallam, 1983; Babior, 1984). Intracellular calcium

also activates phospholipase A_2, stimulating arachidonic acid metabolism. It is possible that the former events occur faster than the mobilization of phospholipids and release of arachidonic acid, thus explaining the lag-time. Certainly the fact that not only do pBPB and NDGA inhibit 5-HPETE production, but also that their ED_{50} values are similar for inhibition of both HAGG- and FMLP-stimulated production of H_2O_2 indicates that a common mechanism of activation is involved.

Although the cyclooxygenase inhibitors are not as potent as phospholipase A_2/lipoxygenase inhibitors at suppressing ROOH production, nonetheless they do have some ability in this direction except for piroxicam. If ROOH is 5-HPETE, this is surprising especially as several investigators have shown that in fact cyclooxygenase inhibitors appear to stimulate metabolism of arachidonic acid via the lipoxygenase pathway by human neutrophils (Harvey et al, 1983; Myers and Siegel, 1983). This is thought to be due either to the shunting of arachidonic acid to the lipoxygenase pathway following inhibition of cyclooxygenase, or to the fact that prostaglandins, which would normally inhibit LTB_4 release, are not present (Ham et al, 1983). The effect of these drugs on ROOH release must be unrelated to their action as cyclooxygenase inhibitors, and neither are they lipoxygenase inhibitors, but rather to another effect of the drugs on these cells. This is more marked with neutrophils as the effects of diclofenac sodium on monocytes were negligible. As well as the inhibitory effects of cyclooxygenase inhibitors on 5-HPETE production, all inhibitors demonstrate activity on H_2O_2 production by neutrophils stimulated with either HAGG or FMLP. Most of the NSAIDS inhibit FMLP-stimulated H_2O_2 production but apparently not HAGG-stimulated H_2O_2 production except for diclofenac sodium. It is obvious from the experiments with the drugs and enzymatically-generated O_2^- that it is unlikely that the inhibitory drugs

are affecting H_2O_2 production merely by scavenging $O_2^{\bullet-}$. Possibly the effects of these drugs on FMLP-stimulated H_2O_2 production may be associated with an inhibition of FMLP binding because, as has been discussed in the Introduction, many NSAIDS have been shown to have this effect (Abita, 1981; Van Dyke et al, 1982). It would have been more satisfactory if time had allowed the use of labelled FMLP to investigate this possibility further. Otherwise it is difficult to explain why, when FMLP is the stimulus, drugs have the ability to inhibit H_2O_2 production, whereas when HAGG is the stimulus, the same drugs stimulate H_2O_2 production. However, the results with HAGG-stimulated cells are really a summation of two opposing events, namely a) inhibition of HAGG-stimulated H_2O_2 production and b) the direct stimulation of H_2O_2 production. Certain drugs are capable of both (a) and (b) but diclofenac sodium only really does (a). Therefore it appears to be more potent than the others but really it is only less directly stimulatory. Various analogues of diclofenac sodium and fenclofenac were used to try to elucidate any structure/activity relationships for the drug effects on neutrophil H_2O_2/5-HPETE production. Considering the diclofenac series, the important factor seems to be the twist in the molecule about the imine link dictated by the position of substitution of atoms (i.e. 2,6) on the ring remote from the acid. Thus, when this twist can be lost, as with the 2-chloro or 2,3-dichloro compounds apparent potency is lost. However, again, this is a summation of inhibitory/stimulatory effects. Any substitution made in the 4-position (Cl or OMe) somewhat enhances potency. Any substitution made in the ring containing the acid moiety has no effect on activity compared with the original compound. This is also the case for substitution in the acid itself (acetic or propionic). This is true for both diclofenac and fenclofenac analogues.

As fenclofenac is approximately twenty times less potent than diclofenac analogue no. 20, and the only difference in the molecule is the ether

linkage in fenclofenac rather than the imine linkage, this link must be important. As the imine group includes the possibility for hydrogen bonding with the carbonyl of the acid group, this would confer on the molecule a more rigid structure for the diclofenac series, keeping the imine group planar with the acid ring. Also the ether link may cause the substituted ring to twist such that it is not in the same plane as the acid ring. This has the same effect on the structure as the 2,6 substitutions in the diclofenac series and so the substitution pattern in the fenclofenac series is less relevant. This explains why the 2-chloro and 2,3-dichloro substituted compounds do not show a lower potency than fenclofenac, whereas the equivalent compounds in the diclofenac series are less potent (or more directly stimulatory) than diclofenac. In common with the diclofenac series, a bulky substitution in the 4-position, as in the isobutyl-substituted compound, increases potency. This may be due to its increased lipophilicity allowing greater association with the cell membrane and perhaps inhibiting FMLP binding. It is also interesting to note that in tests of anti-inflammatory activity in adjuvant arthritis, this analogue was found to be three times more potent than fenclofenac (Atkinson et al, 1983). It is unfortunate that no correlation between potency in these experiments and potency as anti-inflammatory agents could be made with the diclofenac analogues, but such information was not forthcoming from the manufacturers.

There is no obvious similarity in structure between the drugs and drug analogues which directly stimulate neutrophils which might explain this activity. Similarly, there is no obvious correlation between the structures of the drugs which interfere with the phenol red assay, or between these compounds and phenol red. Substitution of CH_3 groups instead of chlorine atoms in the diclofenac series results in inter-

ference with the phenol red assay whereas a similar substitution in the fenclofenac series does not have that effect. The drugs appear to have this direct effect on the assay due to their ability to act as alternative substrates for peroxidase, displacing phenol red and being oxidized themselves. Thus peroxidase must have a very open catalytic site to be able to interact with such a wide variety of structures. The inhibitory effects of these drugs on neutrophil activity could be explained in terms of interactions with the cell membrane, cross-linking proteins and thus preventing mobilization of phospholipids or stimulation of NADPH oxidase. Certainly it has been demonstrated that cross-linking agents interfere with the activation of NADPH oxidase by reacting with amino groups on the cell surface (Aviram, Simons and Babior, 1984). The work with sulphydryl group blockers indicates that free SH groups are necessary for activation of both arachidonic acid metabolism via lipoxygenase and NADPH oxidase. Whether these compounds inhibit activity by preventing mobilization of essential components of the cell membrane, or whether SH groups are a vital structural part of the effector enzymes is not clear, but it does demonstrate that an interaction with certain molecular groups on the cell surface can have substantial effects on cell activity.

The relevance of all this work *in vivo* is questionable because the concentrations of free drug in the plasma or synovial fluid is very much lower than concentrations employed in most experimental studies, as already discussed in the Introduction. As shown here, there is no effect on neutrophil activity by either diclofenac sodium or piroxicam *in vivo* and, in any case, piroxicam *in vitro* has very little effect compared with the other cyclooxygenase inhibitors at comparable concentrations. Both these observations do not agree with work carried

out by Weissmann's group (Abramson et al, 1983) who demonstrated that piroxicam *in vitro* inhibited FMLP-stimulated $O_2^{\bullet-}$ release and piroxicam treatment led to inhibition of FMLP-stimulated production of $O_2^{\bullet-}$ by neutrophils. However this was using a small sample of healthy volunteers (age-range unknown) who were taking the drug for 3 days only. It is possible that this cannot be compared with a study using mainly elderly osteoarthritic patients, where there may have been a problem with compliance. Also any inhibitory effects Weissmann's group observed with piroxicam may be due to its metabolite, if it does not have any effect *in vitro*. An elderly patient will not metabolise the drug as efficiently. It is certainly true that the young early synovitis patients' neutrophils did not produce as much H_2O_2 in response to stimulation with FMLP following piroxicam treatment. The ability of certain drugs to stimulate neutrophil oxidative metabolism is also questionable *in vivo* due to the concentration factor. It is perhaps interesting to note that the two drugs which have this ability at physiologically relevant concentrations, benoxaprofen and fenclofenac, have both been withdrawn for toxicity reasons. It was intended to include fenclofenac in an *in vivo* study with piroxicam and diclofenac to see whether or not its stimulatory activity could be detected, but it was withdrawn before this could be achieved. These stimulatory effects with benoxaprofen have also been reported by Anderson et al (1984), using physiologically relevant concentrations of the drug. They suggest that its pro-oxidant activity may explain its anti-inflammatory activity by inhibiting neutrophil migration.

Anti-inflammatory drugs may exert some effect by acting as scavengers of oxygen radicals rather than by preventing their formation in the first place. Although it has been shown here that it is unlikely any of the drugs themselves can scavenge enzymatically-generated $O_2^{\bullet-}$,

it has been found that copper complexes of various NSAIDS can eliminate $O_2^{\bullet -}$ radicals (Lengfelder, 1984), acting as catalysts of $O_2^{\bullet -}$ dismutation. Moreover such complexes have been isolated in vivo. Having discussed whether or not NSAIDS can affect oxygen-derived free radical production in vivo as opposed to in vitro, does free radical production by neutrophils have a major role in inflammation or RA anyway? Patients with CGD have neutrophils which fail to generate oxygen-derived free radicals, and yet these patients have a normal inflammatory reaction (Roos and Bragt, 1984). Also neutrophils from patients deficient in myeloperoxidase produce more $O_2^{\bullet -}$ but again have normal inflammatory responses (Roos and Bragt, 1984). On the other hand many investigators have reported beneficial effects of superoxide dismutase in human inflammatory diseases and experimental models of inflammation, indicating a role for oxygen-derived free radicals (Goebel, Storck and Neurath, 1981). It should also be remembered that products of lipid peroxidation are present in the plasma and synovial fluid of patients with rheumatoid disease (Rowley et al, 1984) and also that free radical -damaged IgG is found in vivo (Lunec, 1984; Wickens and Dormandy, 1984). Another relevant point is that $O_2^{\bullet -}$ reduces the viscosity of synovial fluid. SOD and catalase protect against this as do several NSAIDS (McCord, 1974; Puig-Parellada and Planas, 1978).

The work performed with neutrophils from patients with PSS indicates that neutrophils from these patients ex vivo are in an activated state, producing significant amounts of H_2O_2 and 5-HPETE. This correlates well with increased expression of Fc receptors. Without performing Scatchard plot analysis, it is not possible to say conclusively whether this is due to increased numbers of receptors, or to an increase in receptor avidity. However, as the shift to the right of the graph showing % rosetting cells against dilution of IgG for patients is most

significant at the very weakest dilutions of IgG, the explanation of increased numbers of receptors is the more likely. In monocytes, increased expression of Fc receptors is indicative of activation and indeed it has been found that RA patients' monocytes carry more Fc receptors than do healthy controls (Carter et.al, 1984). However, no such increase in Fc receptor expression in RA patients' neutrophils can be demonstrated (Minty, 1982) and no increase in activity of RA patients' neutrophils is shown here.

Thus, neutrophils in the microvasculature of PSS patients are producing H_2O_2 which is capable of mediating endothelial cell damage (Weiss et al, 1981) and could contribute significantly to the vascular pathology of this disease. Moreover, the cells are also capable of producing more 5-HPETE, and hence presumably LTB_4, serving to recruit more neutrophils by chemotaxis and encouraging increased adherence and margination, contributing to cell damage (Samuelsson, 1983; Piper, 1984). However, as it has been demonstrated that the proportion of 5-HPETE produced by neutrophils falls with increasing cell numbers, it is debatable whether this is significant. It must also be borne in mind that PSS is not essentially an inflammatory disease and that the involvement of free radicals and/or LTB_4 must be restricted to possible damage to endothelial cells only. As neutrophils from the control group with peripheral vascular disease did not show enhanced activity, it must be assumed that this phenomenon is not indicative of general vascular disease. An interesting patient group to have studied would have been those with rheumatoid vasculitis, where there is damage to the vascular endothelium.

It is interesting to speculate on the stimulus that is activating these patients' neutrophils <u>in vivo</u>. It may be that the patients whose neutrophils were the most active in this study had the highest level of

circulating immune complexes. It has been found that 20% of patients with PSS do have circulating immune complexes (Dr. P. J. Maddison, Personal Communication). However, if immune complexes were responsible for activation of neutrophils, one would expect to see activated neutrophils in patients with RA. Other possible stimuli include LTB_4; some factor from damaged endothelial cells; or proteases, especially as these patients have a functional defect in serum protease inhibitors.

The observation that it is patients with localized disease who appear to have the most active neutrophils, rather than those with the more serious diffuse disease is surprising. A possible explanation is that those with diffuse disease do have more active cells, but these cells are so activated that they remain marginated to the endothelium and the cells collected in the blood sample are the less activated ones. Finding activated neutrophils in the vascular system is unusual and may be relevant in contributing to the vascular pathology of the disease. It may be important to treat patients with PSS with drugs that suppress neutrophil function or that scavenge oxygen-derived free radicals. It is interesting that some patients do benefit from treatment with penicillamine or colchicine.

BIBLIOGRAPHY

B I B L I O G R A P H Y

ABITA J.P., 1981. Indomethacin is a competitive inhibitor of the binding of the chemotactic peptide formyl-met-leu-phe to human polymorphonuclear leukocytes. Agents Actions 11(6-7) 610-611.

ABRAMSON S., EDELSON H., KAPLAN H., GIVEN W., WEISSMANN G., 1983. The neutrophil in rheumatoid arthritis: its role and the inhibition of its activation by non-steroidal anti-inflammatory drugs. Semin. Arthritis Rheum. 13(1) (Suppl. 1) 148-153.

ABRAMSON S., HOFFSTEIN S.T., WEISSMANN G., 1982. Superoxide anion generation by human neutrophils exposed to monosodium urate. Effect of protein adsorption and complement activation. Arthritis Rheum. 25(2) 174-180.

AMBRUSO D.R., JOHNSTON R.B. Jr., 1981. Lactoferrin induces hydroxyl radical production by human neutrophils, neutrophil particulate fractions and an enzymatic generating system. J. Clin. Invest. 67 352-360.

ANDERSON R., LUKEY P.T., NAUDE S.P.E., JOONE G., 1984. Benoxaprofen: a pro-oxidant anti-inflammatory drug? Agents Actions 14(2) 238-246.

ATKINSON D.C., GODFREY K.E., MEEK B., SAVILLE J.F., STILLINGS M.R., 1983(i). Substituted (2-phenoxyphenyl) acetic acids with anti-inflammatory activity 1. J. Med. Chem. 26 1353-1360.

Idem, 1983(ii). Substituted (2-phenoxyphenyl) acetic acids with anti-inflammatory activity 2. J. Med. Chem. 26 1361-1364.

AVIRAM I., SIMONS E.R., BABIOR B.M., 1984. Reversible blockade of the respiratory burst in human neutrophils by a cleavable cross-linking agent. J. Biol. Chem. 259(1) 306-311.

BABIOR B.M., 1978. Oxygen-dependent microbial killing by phagocytes, Part 1. New Eng. J. Med. 298(12) 659-668.
Idem, 1978. Oxygen-dependent microbial killing by phagocytes, Part 2. New Eng. J. Med. 298(13) 721-725.
Idem, 1984. The respiratory burst of phagocytes. J. Clin. Invest. 73 599-601.

BABIOR B.M., CURNETTE J.T., McMURRICH B.J., 1976. The particulate superoxide-forming system from human neutrophils. Properties of the system and further evidence supporting its participation in the respiratory burst. J. Clin. Invest. 58 989-996.

BABIOR B.M., KIPNES R.S., 1977. Superoxide-forming enzyme from human neutrophils. Evidence for a flavin requirement. Blood 50(3) 517-524.

BABIOR B.M., KIPNES R.S., CURNETTE J.T., 1973. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 52 741-744.

BABIOR G.L., ROSIN R.E., McMURRICH B.J., PETERS W.A., BABIOR B.M., 1981. Arrangement of the respiratory burst oxidase in the plasma membrane of the neutrophil. J. Clin. Invest. 67 1724-1728.

BADWEY J.A., CURNETTE J.T., KARNOVSKY M.L., 1979. The enzyme of granulocytes that produces superoxide and peroxide. An elusive pimpernel. New Eng. J. Med. 300(20) 1157-1160.

BADWEY J.A., KARNOVSKY M.L., 1980. Active oxygen species and the functions of phagocytic leukocytes. Ann. Rev. Biochem. 49 695-726.

BAEHNER R.L., GILMAN N., KARNOVSKY M.L., 1970. Respiration and glucose oxidation in human and guinea pig leukocytes. Comparative studies. J. Clin. Invest. 49 692-700.

BAEHNER R.L., MURRMANN S.K., DAVIS J., JOHNSTON R.B. Jr., 1975. The role of superoxide anion and hydrogen peroxide in phagocytosis - associated oxidative metabolic reactions. J. Clin. Invest. 56 571-576.

BAGGIOLINI M., 1982. Proteinases and acid hydrolases of neutrophils and macrophages and the mechanisms of their release. In Advances in Inflammation Research Vol. 3: Rheumatoid Arthritis edited by Ziff M., Velo G.P., Gorini S., Raven Press, New York, USA.

BAKER D.G., DAYER J.M., ROELKE M., SCHUMACHER H.R., KRANE S.M., 1983. Rheumatoid synovial cell morphologic changes induced by a mononuclear cell factor in culture. Arthritis Rheum. 26(1) 8-14.

BAKHLE Y.S., 1983. Synthesis and catabolism of cyclooxygenase products. Brit. Med. Bull. 39(3) 214-218.

BANFORD J.C., BROWN D.H., HAZELTON R.A., McNEIL C.J., SMITH W.E., STURROCK R.D., 1982. Altered thiol status in patients with rheumatoid arthritis. Rheumatol. Int. 2 107-111.

BANNISTER J.V., BANNISTER W.H., HILL H.A.O., MAHOOD J.F., WILLSON R.L., WOLFENDEN B.S., 1980. Does ceruloplasmin dismute superoxide? No. FEBS Lett. 118(1) 127-129.

BANNISTER J.V., BELLAVITE P., DAVOLI A., THORNALLEY P.J., ROSSI F., 1982. The generation of hydroxyl radicals following superoxide production by neutrophil NADPH oxidase. FEBS Lett. 150(2) 300-302.

BEAUCHAMP C., FRIDOVICH I., 1970. A mechanism for the production of ethylene from methional: the generation of hydroxyl radical by xanthine oxidase. J. Biol. Chem. 245 4641-4646.

BECKER E.L., SHOWELL H.J., NACCACHE P.H., SHA'AFI R.I., 1981. The role and interrelationships of Ca^{2+} and arachidonic acid metabolism in stimulated neutrophil functions. Biochem. Acute Allerg. React. 257-278 pub. Liss A.R. New York USA.

BENDER J.G., McPHAIL L., VAN EPPS D.E., 1983. Exposure of human neutrophils to chemotactic factors potentiates activation of the respiratory burst enzyme. J. Immunol. 130(5) 2316-2323.

BERRIDGE M.J., 1984. Inositol triphosphate and diacylglycerol as second messengers. Biochem. J. 220 345-360.

BIELSKI B.H.J., ARUDI R., SUTHERLAND M.W., 1983. A study of the reactivity of $HO_2/O_2^{\cdot-}$ with unsaturated fatty acids. J. Biol. Chem. 258(8) 4759-4761.

BIEMOND P., SWAAK A.J., KOSTER J.F., 1984. Protective factors against oxygen free radicals and hydrogen peroxide in rheumatoid arthritis synovial fluid. Arthritis Rheum. 27(7) 760-765.

BIEMOND P., VAN EIJK H.G., SWAAK, A.J., KOSTER J.F., 1984. Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. Possible mechanism in inflammation diseases. J. Clin. Invest. 73 1576-1579.

BIRD J., LEWIS D.A., 1983. Anti-inflammatory factors in human synovial fluid. Biochem. Pharmacol. 32(5) 936-938.

BLAKE D.R., BACON P.A., EASTHAM E.J., BRIGHAM K., 1980. Synovial fluid ferritin in rheumatoid arthritis. Br. Med. J. 281 715-716.

BLAKE D.R., BLANN A., BACON P.A., FARR M., GUTTERIDGE J.M.C., HALLIWELL B., 1983(i). Ferroxidase and ascorbate oxidase activities of caeruloplasmin in synovial fluid from rheumatoid patients. Clin. Sci. 64 551-553.

BLAKE D.R., HALL N.D., BACON P.A., DIEPPE P.A., HALLIWELL B., GUTTERIDGE J.M.C., 1983(ii). Effect of a specific iron chelating agent on animal models of inflammation. Annals Rheum. Dis. 42 89-93.

BLAKE D.R., HALL N.D., TREBY D.A., HALLIWELL B., GUTTERIDGE J.M.C., 1981. Protection against superoxide and hydrogen peroxide in synovial fluid from rheumatoid patients. Clin. Sci. 61 483-486.

BLAKE D.R., LUNEC J., AHERN M., RING E.F.J., BRADFIELD J.. GUTTERIDGE J.M.C., 1985(i). Effects of intravenous iron dextran on rheumatoid synovitis. Annals Rheum. Dis. 44 183-188.

BLAKE D.R., WINYARD P., SCOTT D.G.I., BRAILSFORD S., BLANN A., LUNEC J., 1985(ii). Endothelial cell cytoxicity in inflammatory vascular diseases - the possible role of oxidised lipoproteins. Annals Rheum. Dis. 44 176-182.

BOTSTEIN G.R., SHERER G.K., LEROY E.C., 1982. Fibroblast selection in scleroderma. An alternative model of fibrosis. Arthritis Rheum. 25(2) 189-195.

BRAGT P.C., 1984. Free radicals as a target of anti-rheumatic drug therapy. Agents Actions 15(1/2) 53-55.

BRIGGS R.T., KARNOVSKY M.L., KARNOVSKY M.J., 1977. Hydrogen peroxide production in chronic granulomatous disease. A cytochemical study of reduced pyridine nucleotide oxidases. J. Clin. Invest. 59 1088-1098.

BUCKINGHAM R.B., PRINCE R.K., RODNAN G., BARNES E.L., 1980. Collagen accumulation by dermal fibroblast cultures of patients with linear localized scleroderma. J. Rheumatol. 7(2) 130-142.

CARP H., JANOFF A., 1979. In vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. J. Clin. Invest. 63 793-797.

CARTER S.D., BOURNE J.T., ELSON C.J., HUTTON C.W., CZUDEK R., DIEPPE P.A., 1984. Mononuclear phagocytes in rheumatoid arthritis: Fc-receptor expression by peripheral blood monocytes. Annals Rheum. Dis. 43 424-429.

CARTER S.D., LESLIE R.G.Q., REEVES W.G., 1982. Human monocyte binding of homologous monomer and complexed IgG. Immunology 46 793-800.

CHWALINSKA-SADOWSKA H., BAUM J., 1976. The effect of D-penicillamine on polymorphonuclear leukocyte function. J. Clin. Invest. 58 871-879.

CHUI P.L., DAVIS P., WONG K., DASGUPTA M., 1983. Superoxide production in neutrophils of patients with rheumatoid arthritis and Felty's syndrome. J. Rheumatol. 10(5) 694-700.

CLANCY R.M., DAHINDEN C.A., HUGLI T.E., 1983. Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by N-formyl-met-leu-phe or complement component C5a is independent of phospholipase activation. Proc. Natl. Acad. Sci. USA. 80 7200-7204.

CLARK R.A., 1982. Chemotactic factors trigger their own oxidative inactivation by human neutrophils. J. Immunol. 129(6) 2725-2728.

CLELAND L.G., BETTS W.H., VERNON-ROBERTS B., BIELICKI J., 1982. Role of iron and influence of anti-inflammatory drugs on oxygen-derived free radical production and reactivity. J. Rheumatol. 9(6) 885-892.

COHEN H.J., CHOVANIEC M.E., 1978. Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for a continuous assay for monitoring superoxide production and for the study of the activation of the generating system. J. Clin. Invest. 61 1081-1087.

COHEN H.J., CHOVANIEC M.E., 1978. Superoxide production by digitonin-stimulated guinea pig granulocytes. The effects of N-ethylmaleimide, divalent cations and glycolytic and mitochondrial inhibitors on the activation of the superoxide generating system. J. Clin. Invest. 61 1088-1096.

COHEN S., JOHNSON A.R., HURD E., 1983. Cytoxicity of sera from patients with scleroderma. Effects on human endothelial cells and fibroblasts in culture. Arthritis Rheum. 26(2) 170-178.

COOK R.A., COUNTER F.T., McCOLLEY J.K., 1976. Use of plastics for suspension culture vessels. Methods Cell Biol. 14 113-24.

CRONSTEIN B.N., KRAMER S.B., WEISSMANN G., HIRSCHHORN R., 1983. Adenosine: a physiological modulator of superoxide anion generation by human neutrophils. J. Exp. Med. 158 1160-1177.

CUNNINGHAM C.C., DECHATELET L.R., SPACH P.I., PARCE J.W., THOMAS M.J., LEES C.J., SHIRLEY P.S., 1982. Identification and quantitation of electron-transport components in human polymorphonuclear neutrophils. Biochim. Biophys. Acta 682 430-435.

DAHLEN S-E., BJORK J., HEDQVIST P., ARFORS K-E., HAMMARSTROM S., LINDGREN J-A., SAMUELSSON B., 1981. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: In vivo effects with relevance to the acute inflammatory response. Proc. Natl. Acad. Sci. USA. 78(6) 3887-3891.

DANCEY J.T., DEUBELBEISS K.A., HARKER L.A., FINCH C.A., 1976. Neutrophil kinetics in man. J. Clin. Invest. 58 705-715.

DAVIDSON E.M., RAE S.A., SMITH M.J.H., 1983. Leukotriene B_4, a mediator of inflammation present in synovial fluid in rheumatoid arthritis. Annals Rheum. Dis. 42 677-679.

DECHATELET L.R., 1978. Initiation of the respiratory burst in human polymorphonuclear neutrophils. A critical review. J. Retic. Soc. 24(1) 73-91.

DEL MAESTRO R., THAW H., BJORK J., PLANKER M., ARFORS K., 1980. Free radicals as mediators of tissue injury. Acta. Physiol. Scand. Suppl. 492 43-57.

DELLA BIANCA V., BELLAVITE P., DE TOGNI P., FUMARULO R., ROSSI F. Studies on stimulus-response coupling in human neutrophils: I. Role of monovalent cations in the respiratory and secretory response to N-FMLP. Biochim. Biophys. Acta 755 497-505.

DE RIEMER S.A., STRONG J.A., ALBERT K.A., GREENGARD P., KACZMAREK L.K., 1985. Enhancement of calcium current in Aplysia neurones by phorbol ester and protein kinase C. Nature 313 313-316.

DE TOGNI P., DELLA BIANCA V., BELLAVITE P., GRZESKOWIAK M., ROSSI F., 1983. Studies on stimulus-response coupling in human neutrophils: II. Relationships between the effects of changes of external ionic composition on the properties of N-FMLP receptors and on the respiratory and secretory responses. Biochim. Biophys. Acta 755 506-513.

DEWALD B., BAGGIOLINI M., CURNETTE J., BABIOR B.M., 1979. Subcellular localization of the superoxide-forming enzyme in human neutrophils. J. Clin. Invest. 63 21-29.

DIXIT R., MUKHTAR H., BICKERS D.R., 1982. Evidence that lipid peroxidation in microsomal membranes of epidermis is associated with generation of hydrogen peroxide and singlet oxygen. Biochem. Biophys. Res. Comm. 105(2) 546-552.

DORMANDY T.L., 1978. Free-radical oxidation and antioxidants. Lancet Mar 25. 647-650.

DRI P., CRAMER R., SORANZO M.R., MIOTTI V., MENEGAZZI R., PATRIARCA P., 1984. Influence of myeloperoxidase (MPO) on neutrophil functions. Studies with MPO-deficient neutrophils (PMN). Agents Actions 15(1/2) 43-44.

EDELSON H.S., KAPLAN H.B., KORCHAK H.M., SMOLEN J.E., WEISSMANN G., 1982. Dissociation by piroxicam of degranulation and superoxide anion generation from decrements in chlortetracycline fluorescence of activated human neutrophils. Biochem. Biophys. Res. Comm. 104(1) 247-253.

ELSBACH P., 1974. Phagocytosis. In, The Inflammatory Process, Vol. 1 edited by Zweifach B.W., Grant L. and McCluskey R.T., Wew York, Academic Press (2nd Edition) pp 363-408.

ENGLBERGER W., BITTER-SUERMANN D., HADDING U., 1984. Involvement of phospholipid metabolism in the events of the oxidative burst of granulocytes. Agents Actions 15(1/2) 31-33.

FANTONE J.C., KINNES D.A., 1983. Prostaglandin E_1 and prostaglandin I_2 modulation of superoxide production by human neutrophils. Biochem. Biophys. Res. Comm. 113(2) 506-512.

FINCH W.R., RODNAN G.P., BUCKINGHAM R.B., PRINCE R.K., WINKELSTEIN A., 1980. Bleomycin-induced scleroderma. J. Rheumatol. 7 651-659.

FLOWER R.J., BLACKWELL G.J., 1976. The importance of phospholipase A_2 in prostaglandin biosynthesis. Biochem. Pharmacol. 25 285-291.

FOOTE C.S., 1976. in Free Radicals In Biology edited by Pryor W.A. 2 : 85-124 Academic Press, New York.

FRANSON R., WEISS J., MARTIN L., SPITZNAGEL J.K., ELSBACH P., 1977. Phospholipase A activity associated with the membranes of human polymorphonuclear leukocytes. Biochem. J. 167 839-841.

FRIDOVICH I., 1978. The biology of oxygen radicals. The superoxide radical is an agent of oxygen toxicity; superoxide dismutases provide an important defense. Science 201 875-880.

FRIES L.F., MULLINS W.W., CHO K.R., PLOTZ P.H., FRANK M.M., 1984. Monocyte receptors for the Fc portion of IgG are increased in systemic lupus erythematosus. J. Immunol. 132(2) 695-700.

GABIG T.G., SCHERVISH E.W., SANTINGA J.T., 1982. Functional relationship of the cytochrome b to the superoxide-generating oxidase of human neutrophils. J. Biol. Chem. 257(8) 4114-4119.

GALE R., BERTOUCH J.V., BRADLEY J., ROBERTS-THOMSON P.J., 1983. Direct activation of neutrophil chemiluminescence by rheumatoid sera and synovial fluid. Annals Rheum. Dis. 42 158-162.

GALE R., BERTOUCH J.V., GORDON T.P., BRADLEY J., ROBERTS-THOMSON P.J., 1984. Neutrophil activation by immune complexes and the role of rheumatoid factor. Annals Rheum. Dis. 43 34-39.

GALVAN L., HUANG C-H., PRESTAYKO A.W., STOUT J.T., EVANS J.E., CROOKE S.T., 1981. Inhibition of bleomycin-induced DNA breakage by superoxide dismutase. Cancer Res. 41 5103-5106.

GIORDANO G.F., LICHTMAN M.A., 1973. The role of sulfydryl groups in human neutrophil adhesion, movement and particle ingestion. J. Cell. Physiol. 82 387-396.

GODDARD D.H., KIRK A.P., KIRWAN J.P., JOHNSON G.D., HOLBOROW E.J., 1984. Impaired polymorphonuclear leukocyte chemotaxis in rheumatoid arthritis. Annals Rheum. Dis. 43 151-156.

GOEBEL K-M., STORCK U., NEURATH F., 1981. Intrasynovial orgotein therapy in rheumatoid arthritis. Lancet 1015-1017.

GOETZL E.J., 1975. Plasma and cell-derived inhibitors of human neutrophil chemotaxis. Ann. N.Y. Acad. Sci. 256 210-221.

GOLDSTEIN I.M., CERQUEIRA M., LIND S., KAPLAN H.B., 1977. Evidence that the superoxide-generating system of human leukocytes is associated with the cell surface. J. Clin. Invest. 59 249-254.

GOLDSTEIN I.M., HORN J.K., KAPLAN H.B., WEISSMANN G., 1974. Calcium-induced lysozyme secretion from human polymorphonuclear leukocytes. Biochem. Biophys. Res. Comm. 60(2) 807-812.

GOLDSTEIN I.M., KAPLAN H.B., EDELSON H.S., WEISSMANN G., 1979. Ceruloplasmin : a scavenger of superoxide anion radicals. J. Biol. Chem. 254(10) 4040-4045.

GOLDSTEIN I.M., KAPLAN H.B., EDELSON H.S., WEISSMANN G., 1982. Ceruloplasmin: an acute phase reactant that scavenges oxygen-derived free radicals. Annals N.Y. Acad. Sci. 77 368-379.

GOLDSTEIN I.M., ROOS D., KAPLAN H.B., WEISSMANN G., 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. J. Clin. Invest. 56 1155-1163.

GRANT L., 1974. The sticking and emigration of white blood cells in inflammation. In, The Inflammatory Process, Vol. II edited by Zweifach B.W., Grant L., and McCluskey R.T., New York, Academic Press (2nd edition) pp 205-249.

GREEN T.R., WU D.E., WIRTZ M.K., 1983. The O_2^- generating oxidoreductase of human neutrophils: evidence of an obligatory requirement for calcium and magnesium for expression of catalytic activity. Biochem. Biophys. Res. Comm. 110 3973-3978.

GREENWALD R.A., 1981. Oxyradicals and connective tissue - editorial J. Rheumatol. 8(2) 185-187.

GREENWALD R.A., 1981. Effect of oxygen-derived free radicals on connective tissue macromolecules: inhibition by copper-penicillamine complex. J. Rheumatol. 8(7) 9-13.

GREENWALD R.A., MOY W.W., 1980. Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheum. 23(4) 455-463.

GUTTERIDGE J.M.C., 1982. The role of superoxide and hydroxyl radicals in phospholipid peroxidation catalysed by iron salts. FEBS Lett. 150(2) 454-458.

GUTTERIDGE J.M.C., 1982. Fate of oxygen free radicals in extracellular fluids. Biochem. Soc. Trans. 10 72-73.

GUTTERIDGE J.M.C., 1983. Antioxidant properties of caeruloplasmin towards iron - and copper-dependent oxygen radical formation. FEBS Lett. 157(1) 37-40.

GUTTERIDGE J.M.C., BEARD A.P.C., QUINLAN G.J., 1983. Superoxide-dependent lipid peroxidation problems with the use of catalase as a specific probe for Fenton-derived hydroxyl radicals. Biochem. Biophys. Res. Comm. 117(3) 901-907.

GUTTERIDGE J.M.C., RICHMOND R., HALLIWELL B., 1979. Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. Biochem. J. 184 469-472.

GUTTERIDGE J.M.C., RICHMOND R., HALLIWELL B., 1980. Oxygen free-radicals and lipid peroxidation: inhibition by the protein ceruloplasmin. FEBS Lett. 112(2) 269-272.

GUTTERIDGE J.M.C., ROWLEY D.A., HALLIWELL B., 1982. Superoxide-dependent formation of hydroxyl radicals and lipid peroxidation in the presence of iron salts. Biochem. J. 206 605-609.

HALL N.D., 1978. Leukocyte migration inhibition with IgG-IgG complexes and rabbit IgG fragments in patients with rheumatoid arthritis. Clin. Exp. Immunol. 34 219-225.

HALL N.D., BLAKE D.R., BACON P.A., 1982. Serum sulphydryl levels in early synovitis. J. Rheumatol. 9 593-596.

HALL N.D., MASLEN C.L., BLAKE D.R., 1984. The oxidation of serum sulphydryl groups by hydrogen peroxide secreted by stimulated phagocytic cells in rheumatoid arthritis. Rheumatol. Int. 4 35-38.

HALLIWELL B., 1978. Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates. Is it a mechanism for hydroxyl radical production in biochemical systems? FEBS Lett. 92(2) 321-326.

HALLIWELL B., 1982. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts is a feasible source of hydroxyl radicals in vivo. Biochem. J. (Letters) 205 461-462.

HALLIWELL B., GUTTERIDGE J.M.C., 1984. Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. Lancet Jun 23 1396-1397.

HAM E.A., SODERMAN D.D., ZANETTI M.E., DOUGHERTY H.W., McCAULEY E., KUEHL F.A. Jr., 1983. Inhibition by prostaglandins of leukotriene B_4 release from activated neutrophils. Proc. Natl. Acad. Sci. USA. 80 4349-4353.

HARLAN J.M., CALLAHAN K.S., 1984. Role of hydrogen peroxide in the neutrophil-mediated release of prostacyclin from cultured endothelial cells. J. Clin. Invest. 74 442-448.

HARVEY J., PARISH H., HO P.P.K., BOOT J.R., DAWSON W., 1983. The preferential inhibition of 5-lipoxygenase product formation by benoxaprofen. J. Pharm. Pharmacol. 35 44-45.

HASHIMOTO Y., ZIFF M., HIRD E.R., 1982. Increased endothelial cell adherence, aggregation and superoxide generation by neutrophils incubated in S.L.E. and Felty's syndrome sera. Arthritis Rheum. 25(12) 1409-1417.

HED J., STENDAHL O., 1982. Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils. Immunology 45 727-736.

HED J., STENDAHL O., SUNDQVIST T., 1983. Differences in the association between the oxidase-dependent activity and plasma membrane receptors for IgG, C3b and concanavalin A of human neutrophils. FEBS Lett. 152(2) 212-216.

HENDERSQN W.R., KLEBANOFF S.J., 1983. Leukotriene production and inactivation by normal, chronic granulomatous disease and myeloperoxidase-deficient neutrophils. J. Biol. Chem. 258(22) 13522-13527.

HENSON P.M., 1976. Membrane receptors on neutrophils. Immunol. Commun. 5(9) 757-774.

HENSON P.M., OADES Z.G., 1975. Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates. Secretion of granule constituents and increased oxidation of glucose. J. Clin. Invest. 56 1053-1061.

HERLIN T., SAND PETERSEN C., ESMANN V., 1978. The role of calcium and cyclic adenosine 3', 5'-monophosphate in the regulation of glycogen metabolism in phagocytozing human polymorphonuclear leukocytes. Biochim. Biophys. Acta 542 63-76.

HIGGS G.A., 1984. The effects of lipoxygenase inhibitors in anaphylactic and inflammatory responses in vivo. Prostaglandins Leuk. Med. 13 89-92.

HIGG G.A., VANE J.R., 1983. Inhibition of cyclooxygenase and lipoxygenase. Brit. Med. Bull. 39(3) 265-270.

HILLER K-O., WILSON R.L., 1983. Hydroxyl-free radicals and anti-inflammatory drugs: biological inactivation studies and reaction rate constants. Biochem. Pharmacol. 32(13) 2109-2111.

HIRSCH J.G., 1974. Neutrophil leukocytes. In, The Inflammatory Process, Vol. I, edited by Zweifach B.W., Grant L. and McCluskey R.T., New York, Academic Press (2nd Edition) pp 411-447.

HOFFMAN A., AUTOR A., 1982. Effect of cyclooxygenase inhibitors and protease inhibitors on phorbol-induced stimulation of oxygen consumption and superoxide production by rat pulmonary macrophages. Biochem. Pharmacol. 31(5) 775-780.

HOLLAND C., JAYSON M.I.V., 1981. Rheumatoid arthritis. Med. Int. 1(10) 427-433.

HOVESTADT I., FERBER E., 1984. The effect of free fatty acids and acyl-CoA's on the activity of NAD(P)H-oxidase as measured by chemiluminescence of subcellular fractions of bone marrow macrophages. Agents Actions 15(1/2) 40-41.

IGNARRO L.J., CECH S.Y., 1976. Bidirectional regulation of lysosomal enzyme secretion and phagocytosis in human neutrophils by guanosine 3', 5'-monophosphate and adenosine 3', 5'-monophosphate. Proc. Soc. Exp. Biol. Med. 151 448-452.

IGNARRO L.J., GEORGE W.J., 1974. Mediation of immunologic discharge of lysosomal enzymes from human neutrophils by guanosine 3', 5'-monophosphate requirement of calcium and inhibition by adenosine 3', 5'-monophosphate. J. Exp. Med. 140 225-238.

ISHIZAKA K., ISHIZAKA T., LEE E.H., FUNDENBERG H.H., 1965. Immuno-chemical properties of human A isohaemagglutinin. 1. Comparisons with G and M globulin antibodies. J. Immunol. 95 197.

JASIN H.E., 1983. Generation of IgG aggregates by the myeloperoxidase - hydrogen peroxide system. J. Immunol. 130(4) 1918-1923.

JIMENEZ S.A., 1983. Cellular immune dysfunction and the pathogenesis of scleroderma. Sem. Arthritis Rheum. 13(1) Suppl. 1 104-113.

JOHNSTON R.B.Jr., KEELE B.B., MISRA H.P., LEHMEYER J.E., WEBB L.S., BAEHNER R.L., RAJAGOPALAN K.V., 1975. The role of superoxide anion generation in phagocytic bactericidal activity. Studies with normal and chronic granulomatous disease leukocytes. J. Clin. Invest. 55 1357-1372.

JOHNSTON R.B.Jr., LEYMEYER J.E., 1976. Elaboration of toxic oxygen by-products by neutrophils in a model of immune complex disease. J. Clin. Invest. 57 836-841.

JOHNSTON R.B. Jr., LEHMEYER J.E., GUTHRIE L.A., 1976. Generation of superoxide anion and chemiluminescence by human monocytes during phagocytosis and on contact with surface-bound immunoglobulin G. J. Exp. Med. 143 1551-1556.

JONES H.P., GHAI G., PETRONE W.F., McCORD J.M., 1982. Calmodulin-dependent stimulation of the NADPH oxidase of human neutrophils. Biochim. Biophys. Acta 714 152-156.

JONES C.J.P., MORRIS K.J., JAYSON M.I.V., 1983. Prednisolone inhibits phagocytosis by polymorphonuclear leucocytes via steroid receptor mediated events. Annals Rheum. Dis. 42 56-62.

KAHALAH M.B., LEROY E.C., 1983. Endothelial injury in scleroderma. A protease mechanism. J. Lab. Clin. Med. 101 553-560.

KAPLAN H.B., EDELSON H.S., FRIEDMAN R., WEISSMANN G., 1982. The roles of degranulation and superoxide anion generation in neutrophil aggregation. Biochim. Biophys. Acta 721 55-63.

KELLOGG E.W. III, FRIDOVICH I., 1975. Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J. Biol. Chem. 250(22) 8812-8817.

KELLOGG E.W. III, FRIDOVICH I., 1977. Liposome oxidation and erythrocyte lysis by enzymatically generated superoxide and hydrogen peroxide. J. Biol. Chem. 252(19) 6721-6728.

KITAGAWA S., TAKAKU F., SAKAMOTO S., 1980. Evidence that proteases are involved in superoxide production by human polomorphonuclear leukocytes and monocytes. J. Clin. Invest. 65 74-81.

KLEBANOFF S.J., 1967. A peroxidase-mediated anti-microbial system in leukocytes. J. Clin. Invest. 46 1078.

KLEBANOFF S.J., 1982. The iron-H_2O_2-iodide cytoxic system. J. Exp. Med. 156 1262-1267.

KORCHAK H.M., EISENSTAT B.A., HOFFSTEIN S.T., DUNHAM P.B., WEISSMANN G., 1980. Anion channel blockers inhibit lysosomal enzyme secretion from human neutrophils without affecting generation of superoxide anion. Proc. Natl. Acad. Sci. USA. 77(5) 2721-2725.

KORCHAK H.M., RUTHERFORD L.E., WEISSMANN G., 1984. Stimulus response coupling in the human neutrophil. I. Kinetic analysis of changes in calcium permeability. J. Biol. Chem. 259(7) 4070-4075.

KORCHAK H.M., VIENNE K., RUTHERFORD L.E., 1984. Stimulus response coupling in the human neutrophil. II. Temporal analysis of changes in cytosolic calcium and calcium efflux. J. Biol. Chem. 259(7) 4076-4082.

KORCHAK H.M., WEISSMANN G., 1978. Changes in membrane potential of human granulocytes antecede the metabolic responses to surface stimulation. Proc. Natl. Acad. Sci. USA. 75 3818-3822.

KREISLE R.A., PARKER C.W., 1983. Specific binding of leukotriene B_4 to a receptor on human polymorphonuclear leukocytes. J. Exp. Med. 157 628-641.

LEHRER R.I., COHEN L., 1981. Receptor-mediated regulation of superoxide production in human neutrophils stimulated by phorbol myristate acetate. J. Clin. Invest. 68 1314-1320.

LENGFELDER E., 1984. Can anti-inflammatory drugs act as scavengers of oxygen radicals? Agents Actions 15(1/2) 56.

LENTNEK A.L., SCHREIBER A.D., MACGREGOR R.R., 1976. The induction of augmented granulocyte adherence by inflammation. Mediation by a plasma factor. J. Clin. Invest. 57 1098-1103.

LEW P.D., DAYER J.M., WOLLHEIM C.B., POZZAN T., 1984. Effect of leukotriene B_4, prostaglandin E_2 and arachidonic acid on cytosolic-free calcium in human neutrophils. FEBS Lett. 166(1) 44-48.

LISIEWICZ J., 1980. Human neutrophils. Published by The Charles Press Publishers Inc., Bowie, Maryland, USA.

LORBER A., PEARSON C.M., MEREDITH W.L., GANTZ-MANDELL L.E., 1964. Serum sulphydryl determinations and significance in connective tissue diseases. Ann. Intern. Med. 61 423-434.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L., RANDALL R.J., 1951. Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193 265-275.

LUNEC J., 1984. Free-radical mediated aggregation of human IgG stimulates neutrophils to generate superoxide radicals. Agents Actions 15(1/2) 37-38.

LUNEC J., HALLORAN S.P., WHITE A.G., DORMANDY T.L., 1981. Free radical oxidation (peroxidation) products in serum and synovial fluid in rheumatoid arthritis. J. Rheumatol. 8(2) 233-245.

MACGREGOR R.R., 1976. The effect of anti-inflammatory agents and inflammation on granulocyte adherence. Evidence for regulation by plasma factors. Am. J. Med. 61 597-607.

MATSUMOTO T., TAKESHIGE K., MINAKAMI S., 1979. Inhibition of phagocytic metabolic changes of leukocytes by an intracellular calcium-antagonist 8-(N, N-diethylamino)-octyl-3,4,5 trimethoxybenzoate hydrochloride. Biochem. Biophys. Res. Comm. 88(3) 974-979.

McCORD J.M., 1974. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. Science 185 529-531.

McCORD J.M., 1980. Oxygen free radicals: release and activities. A superoxide-activated chemotactic factor and its role in the inflammatory process. Agents Actions 10(6) 522-527.

McCORD J.M., DAY E.D., 1978. Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS Lett. 86(1) 139-142.

McCORD J.M., FRIDOVICH I., 1969. Superoxide dismutase - an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244(22) 6049-6055.

McCORD J.M., WONG K., STOKES S., PETRONE W., ENGLISH D., 1980. Superoxide and inflammation: a mechanism for the anti-inflammatory activity of superoxide dismutase. Acta Physiol. Scand. Suppl. 492 25-30.

McGUIRE M.K.B., MEATS J.E., GLOSWORTH N.M., HARVEY L., MURPHY G., RUSSELL R.G.G., REYNOLDS J.J., 1982. Properties of rheumatoid and normal synovial tissue in vitro and cells derived from them. Rheumatol. Int. 2 113-120.

McPHAIL L.C., DECHATELET L., SHIRLEY P., 1976. Further characterization of NADPH oxidase activity of human polymorphonuclear leukocytes. J. Clin. Invest. 58 774-780.

McPHAIL L.C., HENSON P.M., JOHNSTON J.B. Jr., 1981. Respiratory burst enzyme in human neutrophils. Evidence for multiple mechanisms of activation. J. Clin. Invest. 67 710-716.

McPHAIL L.C., SNYDERMAN R., 1983. Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemo-attractants and other soluble stimuli. J. Clin. Invest. 72 192-200.

MELLO FILHO A.C., MENEGHINI R., 1984. In vivo formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction. Biochim. Biophys. Acta 781 56-63.

MINTY C.A., 1982. University of Bath Ph.D. thesis.

MONBOISSE J-C., BRAQUET P., BOREL J.P., 1984. Oxygen-free radicals as mediators of collagen breakage. Agents Actions 15(1/2) 49-50.

MOREHOUSE L.A., TIEN M., BUCHER J.R., AUST S.D., 1983. Effect of hydrogen peroxide on the initiation of microsomal lipid peroxidation. Biochem. Pharmacol. 32(1) 123-127.

MOTOHASHI N., MORI I., 1983. Superoxide-dependent formation of hydroxyl radical catalyzed by transferrin. FEBS Lett. 157(1) 197-199.

MOTTOLA C., ROMEO D., 1982. Calcium movement and membrane potential changes in the early phase of neutrophil activation by phorbol myristate acetate. A study with ion-selective electrodes. J. Cell. Biol. 93 129-134.

MOVAT H.Z., 1979. Tissue injury and inflammation induced by immune complexes: the critial role of the neutrophil leukocyte. Exp. Mol. Path. 31 201-210.

MYERS R.F., SIEGEL M.I., 1983. Differential effects of anti-inflammatory drugs on lipoxygenase and cyclo-oxygenase activities of neutrophils from a reverse passive arthus reaction. Biochem. Biophys. Res. Comm. 112(2) 586-594.

NACCACHE P.H., MOLSKI T.F.P., BORGEAT P., SHA'AFI R.I., 1984. Mechanism of action of leukotriene B_4: intracellular calcium redistribution in rabbit neutrophils. J. Cell. Physiol. 118 13-18.

NACCACHE P.H., SHA'AFI R.I., 1983. Arachidonic acid, leukotriene B_4 and neutrophil activation. Ann. N.Y. Acad. Sci. 414 125-139.

NACCACHE P.H., SHOWELL H.J., BECKER E.L., SHA'AFI R.I., 1977. Transport of sodium, potassium and calcium across rabbit polymorphonuclear leukocyte membranes. Effect of chemotactic factor. J. Cell. Biol. 73 428-444.

NACCACHE P.H., SHOWELL H.J., BECKER E.L., SHA'AFI R.I., 1979. Involvement of membrane calcium in the response of rabbit neutrophils to chemotactic factors as evidenced by the fluorescence of chlorotetracycline. J. Cell. Biol. 83 179-186.

NAKAMURA M., BAXTER C.R., MASTERS B.S.S., 1981. Simultaneous demonstration of phagocytosis - connected oxygen consumption and corresponding NAD(P)H oxidase activity: direct evidence for NADPH as the predominant electron donor to oxygen in phagocytizing human neutrophils. Biochem. Biophys. Res. Comm. 98(3) 743-751.

NELSON R.D., FIEGEL V.D., HERRON M.J., GRACYK J.M., BAUMAN M.P., McCORMACK R.T., SIMMONS R.L., 1980. Chemotactic deactivation of human neutrophils: role of stimulation of hexose monophosphate shunt activity in non-specific deactivation. Acta Physiol. Scand. Suppl. 492 31-41.

NIEDEL J.E., KUHN L.J., VANDENBARK G.R., 1983, 1983. Phorbol diester receptor copurifies with protein kinase C. Proc. Natl. Acad. Sci. USA. 80 36-40.

NIWA Y., MIYAKE S., SAKANE T., SHINGU M., YOKOYAMA M., 1982. Autooxidative damage in Behcet's disease - endothelial cell damage following the elevated oxygen radicals generated by stimulated neutrophils. Clin. Exp. Immunol. 49 247-255.

NIWA Y., SAKANE T., SHINGU M., YOKOYAMA M., 1983. Effect of stimulated neutrophils from the synovial fluid of patients with rheumatoid arthritis on lymphocytes - a possible role of increased oxygen radicals generated by the neutrophils. J. Clin. Immunol. 3(3) 228-240.

OYANAGUI Y., 1976. Inhibition of superoxide anion production in macrophages by anti-inflammatory drugs. Biochem. Pharmacol. 25 1473-1480.

OYANAGUI Y., 1978. Inhibition of superoxide anion production in non-stimulated guinea pig peritoneal exudate cells by anti-inflammatory drugs. Biochem. Pharmacol. 27 777-782.

PALMBLAD J., 1984. The role of granulocytes in inflammation. Scand. J. Rheumatol. 13 163-172.

PALMBLAD J., GYLLENHAMMAR H., AKE LINDGREN J., MALMSTEN C.L., 1984. Effects of leukotrienes and F-met-leu-phe on oxidative metabolism of neutrophils and eosinophils. J. Immunol. 132(6) 3041-3045.

PALMER R.M.J., SALMON J.A., 1983. Release of leukotriene B_4 from human neutrophils and its relationship to degranulation induced by FMLP, STZ and the ionophore A23187. Immunology 50 65-73.

PANAYI G.S., 1980. Rheumatoid Arthritis. In, Essential Rheumatology for Nurses and Therapists. Edited by Panayi G.S., Cassell Ltd., London.

PERIANIN A., LABRO M.T., HAKIM J., 1982. Chemokinetic activity of N-formyl-methionyl-leucyl-phenylalanine on human neutrophils and its modulation by phenylbutazone. Biochem. Pharmacol. 31(19) 3071-3076.

PERTOFT H., BACK O., LINDAHL-KIESSLING K., 1968. Separation of various blood cells in colloidal silica-polyvinylpyrrolidone gradients. Exp. Cell. Res. 50 355-368.

PERTOFT H., RUBIN K., KJELLEN L., LAURENT T., KLINGEBORN B., 1977. The viability of cells grown or centrifuged in a new density gradient medium, Percoll (TM). Exp. Cell. Res. 110 449-457.

PICK E., KEISARI Y., 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Meth. 38 161-170.

PICK E., KEISARI Y., 1981. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages-induction by multiple non-phagocytic stimuli. Cell. Immunol. 59 301-318.

PICK E., MIZEL D., 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J. Immunol. Meth. 46 211-227.

PIPER P.J., 1984. Formation and actions of leukotrienes. Physiol. Rev. 64(2) 744-761.

PRESCOTT S.M., ZIMMERMAN G.A., SEEGER A.R., 1984. Leukotriene B_4 is an incomplete agonist for the activation of human neutrophils. Biochem. Biophys. Res. Comm. 122(2) 535-541.

PUIG-PARELLADA P., PLANAS J.M., 1978. Synovial fluid degradation induced by free radicals. In vitro action of several free radical scavengers and anti-inflammatory drugs. Biochem. Pharmacol. 27 535-537.

QUESENBERRY P., LEVITT L., 1979. Hematopoietic stem cells. New Eng. J. Med. 301(14-16) 755-760; 819-823; 868-872.

RASMUSSEN J.M., BRANDSLUND I., LESLIE R.G.Q., SVEHAG S-E., 1983. Quantitative studies of Fc receptors on human monocytes: characterization by binding of homologous and heterologous monomeric IgG and soluble immune complexes of different composition. Immunology 49 537-544.

REISS M., ROOS D., 1978. Differences in oxygen metabolism of phagocytosing monocytes and neutrophils. J. Clin. Invest. 61 480-488.

RINK T.J., SANCHEZ A., HALLAM T.J., 1983. Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. Nature 305 317-319.

ROMEO D., ZABUCCHI G., ROSSI F., 1973. Reversible metabolic stimulation of polymorphonuclear leukocytes and macrophages. Nature 243 111-112.

ROOS D., 1981. Molecular events during phagocyte stimulation. Scand. J. Rheumatol. Suppl. 40 46-52.

ROOS D., BOT A., VAN SCHAIK M., DEBOER M., DAHA M., 1981. Interaction between human neutrophils and zymosan particles: the role of opsonins and divalent cations. J. Immunol. 126(2) 433-440.

ROOS D., BRAGT P.C., 1984. Is phagocyte radical production relevant to inflammation? Agents Actions 15(1/2) 57-58.

ROOT R.K., METCALF J.A., 1977. H_2O_2 release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of H_2O_2: studies with normal and cytochalasin B treated cells. J. Clin. Invest. 60 1266-1279.

ROOT R.K., METCALF J.A., OSHINO N., CHANCE B., 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation and some regulating factors. J. Clin. Invest. 55 945-955.

ROMEO D., ZABUCCHI G., MIANI N., ROSSI F., 1975. Ion movement across leukocyte plasma membrane and excitation of their metabolism. Nature 253 542-544.

ROSEN H., KLEBANOFF S.J., 1979. Bactericidal activity of a superoxide anion-generating system. A model for the polymorphonuclear leukocyte. J. Exp. Med. 149 27-39.

ROSSI F., BELLAVITE P., BERTON G., DRI P., ZABUCCHI G., 1982. The respiratory burst of phagocytic cells: facts and problems. In, Biochemistry and Function of Phagocytes. Edited by Rossi F., Patriarca P., Plenum Press, New York, USA. pp 283-322.

ROWLEY D., GUTTERIDGE J.M.C., BLAKE D., FARR M., HALLIWELL B., 1984. Lipid peroxidation in rheumatoid arthritis: thiobarbituric acid-reactive material and catalytic iron salts in synovial fluid from rheumatoid patients. Clin. Sci. 66 691-695.

ROWLEY D.A., HALLIWELL B., 1982. Superoxide-dependent formation of hydroxyl radicals in the presence of thiol compounds. FEBS Lett. 138(1) 33-36.

ROWLEY D.A., HALLIWELL B., 1983. Formation of hydroxyl radicals from hydrogen peroxide and iron salts by superoxide - and ascorbate-dependent mechanisms: relevance to the pathology of rheumatoid disease. Clin. Sci. 64 649-653.

RYAN G.B., MAJNO G., 1977. Mediators of inflammation. In, Inflammation. Edited by Baird T.A., The Upjohn Company, Kalamazoo, Michigan, USA. pp 42-57.

SAGONE A.L.Jr., KING G.W., METZ E.N., 1976. A comparison of the metabolic response to phagocytosis in human granulocytes and monocytes. J. Clin. Invest. 57 1352-1358.

SALARI H., BRAQUET P., BORGEAT P., 1984. Comparative effects of indomethacin, acetylenic acids, 15-HETE, nordihydroguaiaretic acid and BW 755C on the metabolism of arachidonic acid in human leukocytes and platelets. Prostaglandins Leuk. Med. 13 53-60.

SALIN M.L., McCORD J.M., 1975. Free radicals and inflammation. Protection of phagocytosing leukocytes by superoxide dismutase. J. Clin. Invest. 56 1319-1323.

SAMUELSSON B., 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 220 568-575.

SBARRA A.J., KARNOVSKY M.L., 1959. The biochemical basis of phagocytosis. 1. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J. Biol. Chem. 234 1355-1362.

SEGAL A.W., 1981. Phagocytic and bactericidal defects in neutrophils. Clin. Immunol. Allergy 1(3) 581-601.

SEGAL A.W., FORTUNATO A., HERD T., 1980. A rapid single centrifugation step method for the separation of erythrocytes, granulocytes and mononuclear cells on continuous density gradients of Percoll. J. Immunol. Meth. 32 209-214.

SEGAL A.W., JONES O.T.G., 1978. Novel cytochrome b system in phagocytic vacuoles of human granulocytes. Nature 276 515-517.

SEGAL A.W., JONES O.T.G., 1979. The subcellular distribution and some properties of the cytochrome b component of the microbicidal oxidase system of human neutrophils. Biochem. J. 182 181-188.

SEGAL A.W., JONES O.T.G., WEBSTER D., ALLISON A.C., 1978. Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatous disease. Lancet 2 446-449.

SEGAL A.W., MESHULAM T., 1979. Production of superoxide by neutrophils: a reappraisal. FEBS Lett. 100(1) 27-32.

SERHAN C.N., BROEKMAN J., KORCHAK H.M., MARCUS A.J., WEISSMANN G., 1982(i). Endogenous phospholipid metabolism in stimulated neutrophils. Differential activation by FMLP and PMA. Biochem. Biophys. Res. Comm. 107(3) 951-958.

SERHAN C.N., LUNDBERG U., WEISSMANN G., SAMUELSSON B., 1984. Formation of leukotrienes and hydroxy acids by human neutrophils and platelets exposed to monosodium urate. Prostaglandins 27(4) 563-81.

SERHAN C.N., RADIN A., SMOLEN J.E., KORCHAK H., SAMUELSSON B., WEISSMANN G., 1982(ii). Leukotriene B_4 is a complete secretagogue in human neutrophils: a kinetic analysis. Biochem. Biophys. Res. Comm. 107(3) 1006-1012.

SHANAHAN W.R. Jr., KORN J.H., 1982. Cytotoxic activity of sera from scleroderma and other connective tissue diseases. Lack of cellular and disease specificity. Arthritis Rheum. 25(12) 1391-1395.

SHERER G.K., JACKSON B.B., LEROY E.C., 1981. Chromosome breakage and sister chromatid exchange frequencies in scleroderma. Arthritis Rheum. 24(11) 1409-1413.

SIMCHOWITZ L., ATKINSON J.P., SPILBERG I., 1982. Stimulation of the respiratory burst in human neutrophils by crystal phagocytosis. Arthritis Rheum. 25(2) 181-188.

SIMCHOWITZ L., MEHTA J., SPILBERG I., 1979. Chemotactic factor-induced generation of superoxide radicals by human neutrophils. Effect of metabolic inhibitors and anti-inflammatory drugs. Arthritis Rheum. 22(7) 755-763.

SIMCHOWITZ L., SPILBERG I., 1979. Generation of superoxide radicals by human peripheral neutrophils activated by chemotactic factor: evidence for the role of calcium. J. Lab. Clin. Med. 93(4) 583-593.

SIMCHOWITZ L., SPILBERG I., 1979. Chemotactic factor-induced generation of superoxide radicals by human neutrophils: evidence for the role of sodium. J. Immunol. 123(5) 2428-2435.

SMITH R.J., IDEN S.S., 1979. Phorbol myristate acetate-induced release of granule enzymes from human neutrophils. Inhibition by the calcium antagonist 8-(N, N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride. Biochem. Biophys. Res. Comm. 91(1) 263-271.

SMITH R.J., IDEN S.S., 1980. Pharmacological modulation of chemotactic factor-elicited release of granule-associated enzymes from human neutrophils. Biochem. Pharmacol. 29 2389-2395.

SMITH R.J., IDEN S.S., 1981. Modulation of human neutrophil superoxide anion generation by the calcium antagonist 8-(N, N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride. J. Reticulo. Endo. Soc. 29(3) 215-225.

SMITH R.J., SUN F.F., BOWMAN B.J., IDEN S.S., SMITH H.W., McGUIRE J.C., 1982. Effect of 6,9-deepoxy-6,9-(phenylimino)-Δ6,8-prostaglandin I_1 (U-60 257), an inhibitor of leukotriene synthesis on human neutrophil function. Biochem. Biophys. Res. Comm. 109(3) 943-949.

SMOLEN J.E., KORCHAK H.M., WEISSMANN G., 1980. Increased levels of cyclic adenosine-3', 5'-monophosphate in human polymorphonuclear leukocytes after surface stimulation. J. Clin. Invest. 65 1077-1085.

SMOLEN J.E., KORCHAK H.M., WEISSMANN G., 1981. The roles of extracellular and intracellular calcium in lysosomal enzyme release and superoxide anion generation by human neutrophils. Biochim. Biophys. Acta 677 512-520.

SMOLEN J.E., WEISSMANN G., 1980. Effects of indomethacin, 5,8,11,14-eicosatetraynoic acid and p-bromophenacyl bromide on lysosomal enzyme release and superoxide anion generation by human polymorphonuclear leukocytes. Biochem. Pharmacol. 29 533-538.

SNYDERMAN R., GOETZL E.J., 1981. Molecular and cellular mechanisms of leukocyte chemotaxis. Science 213 830-837.

SPILBERG I., MEHTA J., 1979. Demonstration of a specific neutrophil receptor for a cell-derived chemotactic factor. J. Clin. Invest. 63 85-88.

SPISANI S., MARANGONI C., DOVIGO L., TRANIELLO S., 1984. Effect of anti-inflammatory agents on neutrophil superoxide production in rheumatoid arthritis. Inflammation 8(1) 45-52.

STEINMAN R.M., COHN Z.A. 1974. Mononuclear phagocytes. In, The Inflammatory Process, Vol. I. Edited by Zweifach B.W., Grant L., and McCluskey R.T., New York, Academic Press (2nd Edition) pp 411-447.

STEVENSON R.D., 1977. Mechanism of anti-inflammatory action of glucocorticosteroids. Lancet 1 225-226.

STOCKER R., RICHTER C., 1982. Involvement of calcium, calmodulin and phospholipase A in the alteration of membrane dynamics and superoxide production of human neutrophils stimulated by phorbol myristate acetate. FEBS Lett. 147(2) 243-246.

STOSSEL T.P., 1974. Phagocytosis. New Eng. J. Med. 290 717-723: 774-780; 833-839.

SUMIMOTO H., TAKESHIGE K., MINAKAMI S., 1984. Superoxide production of human polymorphonuclear leukocytes stimulated by leukotriene B_4. Biochim. Biophys. Acta 803 271-277.

SWEDER VAN ASBECK B., MARX J.J.M., STRUYVENBERG A., HENNY VAN KATS J., VERHOEF J., 1984. Effect of iron (III) in the presence of various ligands on the phagocytic and metabolic activity of human polymorphonuclear leukocytes. J. Immunol. 132(2) 851-856.

TAKEMURA R., WERB Z., 1984. Secretory products of macrophages and their physiological functions. Am. J. Physiol. 246(Cell Physiol. 15) C1-C9

TAKESHIGE K., NABI Z.F., TATSCHECK B., MINAKAMI S., 1980. Release of calcium from membranes and its relation to phagocytic metabolic changes: a fluorescence study on leukocytes loaded with chlortetracycline. Biochem. Biophys. Res. Comm. 95(1) 410-415.

TAUBER A.I., GOETZL E.J., 1981. Inhibition of complement-mediated functions of human neutrophils by impermeant stilbene disulfonic acids. J. Immunol. 126(5) 1786-1789.

TAYLOR G.W., MORRIS H.R., 1983. Lipoxygenase pathways. Brit. Med. Bull. 39(3) 219-222.

THOMAS J., EVANS P.H., 1975. Serum protein changes in coal-workers' pneumoconiosis. Clin. Chim. Acta 60 237-247.

THURMAN R.G., LEY H.G., SCHOLZ R., 1972. Hepatic microsomal ethanol oxidation. Hydrogen peroxide formation and the role of catalase. Eur. J. Biochem. 25 420-430.

TOM W-M., MONTGOMERY M-R., 1980. Bleomycin toxicity. Alterations in oxidative metabolism in lung and liver microsomal fractions. Biochem. Pharmacol. 29 3239-3244.

TREADWAY W., COLLINS R.L., TURNER R.A., DECHATALET L., 1979. Polymorphonuclear leukocyte secretory and metabolic responses to immunoglobulin aggregates. J. Rheumatol. 6(6) 636-644.

TSAN M-F., 1983. Inhibition of neutrophil sulphydryl groups by chloromethyl ketones. A mechanism for their inhibition of superoxide production. Biochem. Biophys. Res. Comm. 112(2) 671-677.

TSAN M-F., NEWMAN B., McINTYRE P.A., 1976. Surface sulphydryl groups and phagocytosis-associated oxidative metabolic changes in human polymorphonuclear leucocytes. Br. J. Haem. 33 189-204.

TSAN M-F., NEWMAN B., CHUSID M., WOLFF S., McINTYRE P.A., 1976. Surface sulphydryl groups and hexose monophosphate pathway activity in resting human polymorphonuclear leucocytes. Br. J. Haem. 33 205-211.

TSAN M-F., BERLIN R.D., 1971. Effect of phagocytosis on membrane transport of non-electrolytes. J. Exp. Med. 134 1016-1035.

TURNER R.A., SEMBLE E.L., JOHNSON J.A., McCRICKARD E.L., TREADWAY W.J., KAUFMANN J.S., 1984. Effects of benoxaprofen on human neutrophil function. J. Rheumatol. 11(3) 265-271.

UGAI K., ISHIKAWA H., HIROHATA K., SHIRANE H., 1983. Interaction of polymorphonuclear leukocytes with immune complexes trapped in rheumatoid articular cartilage. Arthritis Rheum. 26(12) 1434-1441.

VALLEE E., GOUGAT J., NAVARRO J., DELAHAYES J.F., 1979. Anti-inflammatory and platelet anti-aggregant activity of phospholipase-A_2 inhibitors. J. Pharm. Pharmacol. 31 588-592.

VAN DE STADT R.J., VAN DE VOORDE-VISSERS E., FELTKAMP-VROOM T.M., 1980. Metabolic and secretory properties of peripheral and synovial granulocytes in rheumatoid arthritis. Arthritis Rheum. 23(1) 17-23.

VAN DYKE K., PEDEN D., VAN DYKE C., JONES G., CASTRANOVA V., MA J., 1982. Inhibition by non-steroidal anti-inflammatory drugs of luminol-dependent human-granulocyte chemiluminescence and [^{3}H] FMLP binding. Inflammation 6(1) 113-125.

VERNON-ROBERTS B., 1980. Applied anatomy of joints. In, Mason and Currey's Clinical Rheumatology. Edited by Currey H.L.F. (3rd Edition), Pitman Medical Ltd., Tunbridge Wells, Kent.

VICTOR M., WEISS J., KLEMPNER M.S., ELSBACH P., 1981. Phospholipase A_2 activity in the plasma membrane of human polymorphonuclear leukocytes. FEBS Lett. 136(2) 298-300.

WALSH C.E., DECHATELET L.R., CHILTON F.H., WYKLE R.L., WAITE M., 1983. Mechanisms of arachidonic acid release in human polymorphonuclear leukocytes. Biochim. Biophys. Acta 750 32-40.

WALSH C.E., WAITE B.M., THOMAS M.J., DECHATELET L.R., 1981. Release and metabolism of arachidonic acid in human neutrophils. J. Biol. Chem. 256(14) 7228-7234.

WANWIMOLRUK S., BROOKS P.M., BIRKETT D.J., 1983. Protein-binding of non-steroidal anti-inflammatory drugs in plasma and synovial fluid of arthritic patients. Br. J. Clin. Pharmacol. 15 91-94.

WEDMORE C.V., WILLIAMS T.J., 1981. Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature 289 646-650.

WEENING R.S., WEVER R., ROOS D., 1975. Quantitative aspects of the production of superoxide radicals by phagocytizing human granulocytes. J. Lab. Clin. Med. 85(2) 245-252.

WEISS S.J., LoBUGLIO A.F., 1982. Biology of disease: phagocyte-generated oxygen metabolites and cellular injury. Lab. Invest. 47(1) 5-18.

WEISS S.J., YOUNG J., LoBUGLIO A.F., SLIVKA A., NIMEH N.F., 1981. Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. J. Clin. Invest. 68 714-721.

WEISSMANN G., 1982. Activation of neutrophils and the lesions of rheumatoid arthritis. J. Lab. Clin. Med. 100(3) 322-333.

WEISSMANN G., 1983. Pathways of arachidonate oxidation to prostaglandins and leukotrienes. Sem. Arthritis Rheum. 13(1) Suppl. 1 123-129.

WEISSMANN G., SERHAN C.N., KORCHAK H.M., SMOLEN J.E., 1982. Neutrophils: release of mediators of inflammation with special reference to rheumatoid arthritis. Annals N.Y. Acad. Sci. 389 11-24.

WHITE J.R., NACCACHE P.H., MOLSKI T.F.P., BORGEAT P., SHA'AFI R.I., 1983. Direct demonstration of increased intracellular concentration of free calcium in rabbit and human neutrophils following stimulation by chemotactic factor. Biochem. Biophys. Res. Comm. 113(1) 44-50.

WHITESIDE T.L., KUMAGAI Y., ROUMM A.D., ALMENDINGER R., RODNAN G.P., 1983. Supressor cell function and T-lymphocyte subpopulations in peripheral blood of patients with progressive systemic sclerosis. Arthritis Rheum. 26(7) 841-847.

WICKENS D.G., DORMANDY T.L., 1984. Effect of free-radical activity on human gamma-globulin. Agents Actions 15(1/2) 47-48.

WICKENS D.G., GRAFF T.L., LUNEC J., DORMANDY T.L., 1981. Free radical-mediated aggregation of human gamma-globulin. Agents Actions. 11(6/7) 650-651.

WILDFEUER A., 1983. Effects of non-steroidal anti-inflammatory drugs on human leucocytes. Z. Rheumatol. 42 16-20.

WILLIAMS J.D., CZOP J.K., AUSTEN K.F., 1984. Release of leukotrienes by human monocytes on stimulation of their phagocytic receptor for particulate activators. J. Immunol. 132(6) 3034-3040.

WILLIAMS L.T., SNYDERMAN R., RIKE M., LEFKOWITZ R.J., 1977. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc. Natl. Acad. Sci. USA. 74 1204-1208.

WINTERBOURN C.C., 1982. Superoxide-dependent production of hydroxyl radicals in the presence of iron salts. Biochem. J. (Lett.) 205 463.

WRIGHT S.D., SILVERSTEIN S.C., 1982. Tumor-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. J. Exp. Med. 156 1149-1164.

WRIGHT S.D., SILVERSTEIN S.C., 1983. Receptors for C3b and C3b' promote phagocytosis but not the release of toxic oxygen from human phagocytes. J. Exp. Med. 158 2016-2023.

ZIGMOND S.H., 1978. Chemotaxis by polymorphonuclear leukocytes. J. Cell Biol. 77 269-287.

ZVAIFLER N.J., 1971. Immunoreactants in rheumatoid synovial effusions.

J. Exp. Med. 134(3) 276-285.